A COMPARISON OF ADULT AND INFANT TYPES OF GONOCOCCI.*

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The group of organisms known as the gonococcus has been regarded as a heterogenous one from the point of view of the various immunological reactions. Morphologically and culturally the different strains of gonococci seem to be identical, but coincident with the use of the complement fixation test for diagnostic purposes differences in the power of various strains to bind complement were observed when monovalent antigens were used.

Torrey (1) advanced the view of the diversity of strains when studying first the agglutination and precipitin reactions in antigonococcus serum; while his absorption experiments indicated that "an antigonococcus serum may contain in addition to the specific homologous agglutinin several groups of agglutinins which act on various cultures quite independently of one another. At least three groups were found whose major or specific agglutinins are not removed by interabsorption." Later the same author (2) confirmed this view in a study of complement fixation of the same strains. Other investigators obtained essentially similar results, although the division into three main groups was not confirmed generally. Watabiki (3), who employed the test of complement fixation in gonorrheal infections, states that "no distinct differences exist among the various strains of gonococci, but only a comparative difference." A polyvalent antigen was soon introduced for clinical diagnostic purposes. Schwartz and McNeil (4) note that "their experimental work showed that the secret of success of the serum-diagnosis of gonococcus infection in general lay in the use of a polyvalent antigen on account of the fact that the different strains of gonococci seem to differ considerably one from the other."

SOURCES OF CULTURES.

The striking clinical distinction between gonococcal infection in the adult and in young female children suggested, in view of the facts already established by biological tests, a comparative study of gonococci isolated by culture from the two classes of cases.

Two principal groups of cases are recognized clinically. The organisms isolated from these groups were examined and compared in

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their immunological reactions of agglutination and complement fixation. Nine so called adult strains and six infant strains were studied. With one exception all were freshly isolated strains. X, Y, 1, 2, 3, 4, 5, and 6 were adult strains cultivated from cases of acute urethritis with more or less complications; A was one of the original Torrey strains and has been under cultivation for seven years. The six infant strains, BH, G, C, WE, S, and WH, were all isolated from cases of vulvovaginitis in infants and little girls under five years of age. In addition three other strains isolated from cases of ophthalmia were studied, K, L, and CM. K was isolated from a case of purulent ophthalmia in an infant two months of age; L from a girl of eighteen years who had a vaginal discharge; and CM from a boy of fifteen who had no sign or symptom of infection elsewhere and who gave no history of any exposure to contagion. Table I affords a means of easy reference.

TABLE I.

Source of Cultures.

Designation.	Type of strain.	Clinical diagnosis.
X	Adult	Acute urethritis.
Y	Adult	Acute urethritis.
A (Torrey)	Adult	Acute urethritis.
1	Adult	Acute urethritis.
2	Adult	Acute urethritis.
3	Adult	Acute urethritis.
4	Adult	Acute urethritis.
5	Adult	Acute urethritis.
6	Adult	Acute urethritis.
BH	Infant	Vulvovaginitis.
G	Infant	Vulvovaginitis.
C	Infant	Vulvovaginitis.
WE	Infant	Vulvovaginitis.
S	Infant	Vulvovaginitis.
WH	Infant	Vulvovaginitis.
K	Ophthalmic strain	Ophthalmia neonatorum.
L	Ophthalmic strain	Ophthalmia; girl 18 yrs. old, with vaginitis.
CM	Ophthalmic strain	Ophthalmia; boy 15 yrs. old, with no urethritis, and no history of exposure to infection.

Cultivation.—As is well known, the isolation of the gonococcus from the pus in a well marked case of urethritis is comparatively easy, but the isolation of the organism from a case of vulvovaginitis

is more difficult. The easiest method of doing the latter, and one which rarely failed, was by the use of blood agar plates. The culture was taken by means of a platinum loop passed high up in the vaginal canal. If any gonococci were present they usually appeared within eighteen or twenty hours' incubation at 37° C. as the familiar tiny dew-drop colonies. Unless there was an overwhelming number of contaminating organisms in the least heavily planted plates the gonococcus colonies grew first, and in fact several hours before the other organisms appeared. If the plates are made of ascitic agar the initial growth of the contaminating organisms is more rapid than that of the gonococcus, and in eighteen hours practically every plate will be covered with them, making the selection of even probable gonococcal colonies extremely difficult. Once isolated in pure culture, the strains were grown on a stock media of ascitic veal agar.

EXPERIMENTAL PART.

Immunization.—The monovalent sera used in the immunological experiments about to be described were made by immunizing rabbits to the single adult and single infant strains. The inoculations were all intravenous ones of living twenty-four hour cultures, and were made weekly, beginning with one-twentieth or one-tenth of a culture and increasing gradually to one and one-half to two cultures. Usually eight to ten weeks sufficed to produce a serum having a good content of antibodies. The rapid method of immunization advocated by Gay (5) for the typhoid organism proved unsatisfactory when applied to the gonococcus. In each experiment detailed below serum from more than one rabbit immunized to the same strain was used and the series run in duplicate in order that any individual differences in the sera might be avoided.

Agglutination.—When fresh, immune rabbit serum was found to agglutinate its homologous organisms on the average in a dilution of 1 : 1,000, in some instances 1 : 1,500, and the titer of various rabbit sera did not vary appreciably whether the rabbits were immunized with a freshly isolated strain or with the same strain after it had been under cultivation for twelve months. In no instance did the agglutination titer approach the figure of 1 : 700,000 given by

Torrey (1). The highest titer of the Torrey A immune serum with its homologous organism was 1:666. Normal rabbit sera did not agglutinate the organism at all, or in a few instances only, in a dilution of 1:10.

Table II shows the result of the agglutination tests with the serum prepared with the adult organism X, and table III the results with the serum prepared with the infant strain BH.

TABLE II.

Monovalent Rabbit Serum Immune to Adult Gonococcus Strain X. Agglutination Reactions Made at 37° C.

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	1:1,200	1:1,500	Infant serum.
X	−	±	++	++	+++	+++	+++	+++	+++	++	++	++	+	+	
Y	−	±	+	+	++	+++	+++	+++	++	++	++	++			
A	−	±	±	+	+	+	++	++	+	+	±	−			
1	−	+	++	++	+++	+++	+++	++	+	+	+	−			Adult strains.
2	−	±	+	+	+	++	++	+	±	−	−	−			
3	−	+	++	++	++	++	++	++	+	+	+	−			
4	−	±	+	++	+++	++	++	+	−	−	−	−			
5	−	±	+	++	+++	+++	+++	++	±	±	−	−			
6	−	±	+	+	++	++	+	+	−	−	−	−			
BH	−	−	−	+	+	±	−	−	−	−	−	−			
G	−	−	±	+	+	+	±	±	−	−	−	−			
C	−	−	±	±	+	+	+	±	−	−	−	−			Infant strains.
WE	−	−	±	+	+	±	±	−	−	−	−	−			
S	−	−	±	+	+	+	+	±	−	−	−	−			
WH	−	−	±	±	++	+	±	±	−	−	−	−			
K	−	±	±	+	++	++	+	+	±	−	−	−			Ophthalmic strains.
L	−	+	++	++	++	+	+	+	+	±	−	−			
CM	−	+	++	++	+	+	+	+	+	±	−	−			

It will be seen in table II that serum X (adult type) agglutinates its homologous organism and strain Y, also an adult type, equally well in dilutions of 1:1,000, and some specimens of sera agglutinated its homologous organism at 1:1,500. The average titer of other adult strains with this immune adult serum was 1:333, although strain A agglutinated at 1:666 and strains 1 and 3 at 1:800. With infant strains titered against the adult immune serum, however, no agglutination was found in dilutions higher than 1:200, and the average was 1:66 to 1:100. The strains isolated from cases of

gonorrheal ophthalmia approach the adult type in the agglutination reaction with this adult immune serum, although above the dilution of 1 : 100 the reaction is not so marked as with the adult strains. The highest titer obtained with these three ophthalmic strains was 1 : 500 (strains L and CM).

It is interesting to compare the results given in table III with those just noted. Here the same adult strains were titered against a serum prepared with an infant type of organism, BH.

TABLE III.

Monovalent Rabbit Serum Immune to Infant Gonococcus BH. Agglutination Reactions Made at 37° C.

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	1:1,200	1:1,500	Adult serum.
BH	−	±	+	++	++	+++	+++	+++	+++	+++	+++	++	+	±	
G	−	+	+	++	++	++	++	++	+	+	±	±			
C	−	±	±	+	+	++	++	++	++	+	+	±			Infant
WE	−	−	+	++	++	++	++	++	++	++	+	±			strains.
S	−	−	+	++	++	++	+	+	+	±	±	−			
WH	−	±	+	++	++	+	+	+	+	+	−	−			
X	−	±	±	+	+	−	−	−	−	−	−	−			
Y	−	−	±	+	+	±	−	−	−	−	−	−			
A	−	±	±	+	+	+	+	±	−	−	−	−			
1	−	+	+	+	++	+	±	±	−	−	−	−			Adult
2	−	−	±	+	+	±	±	−	−	−	−	−			strains.
3	−	+	+	++	++	++	+	±	−	−	−	−			
4	−	−	±	+	+	+	+	−	−	−	−	−			
5	−	−	−	+	++	+	±	−	−	−	−	−			
6	−	−	+	+	+	+	±	−	−	−	−	−			
K	−	+	+	++	+	+	±	±	−	−	−	−			Ophthal-
L	−	+	+	+	++	+	±	−	−	−	−	−			mic
CM	−	+	+	++	+	±	±	−	−	−	−	−			strains.

The titer of this serum with its homologous organism was high, 1 : 1,000, and with some specimens of sera 1 : 1,200, but the titer of two other infant strains, C and WE, was also high, 1 : 800. The average titer of this infant immune serum with infant strains was 1 : 500. On the other hand, when adult strains were used with this infant immune serum rather striking differences were again brought out. In no case did agglutination occur in dilution of the serum above 1 : 200, and the average was 1 : 100. Strains 1, 3, and 5 were the only ones showing a double plus reaction, and this occurred in

dilutions of 1:66 and 1:100. The three ophthalmic strains when titered against this infant immune serum did not agglutinate in dilutions above 1:100, thus conforming again to the adult type.

As will be seen in tables II and III, a pre-zone of agglutination was present quite regularly. It was seen in all infant strains tested with adult immune serum; strains 1 and 3 never exhibited it when tested with either adult or infant immune sera. The ophthalmic strains practically never showed it; there was a plus-minus reaction of strain K with the lowest dilution of the adult immune serum. When the homologous strains were tested with their own sera there was a plus-minus reaction in the lowest dilution, suggesting a pre-zone when compared with the plus reaction in the succeeding higher dilution, yet not as definite a one as with other strains or when the two strains X (adult) and BH (infant) were crossed with the opposite serum.

TABLE IV.

Monovalent Rabbit Serum Immune to Adult Strain Y. Agglutination Reactions Made at 37° C.

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	Adult serum
Y	−	±	+	++	+++	+++	+++	+++	+++	++	++	++	
X	−	−	+	++	+++	+++	+++	++	++	++	++	++	Adult
1	−	+	+	++	+++	+++	++	++	+	−	−	−	strains.
4	−	±	+	+	++	+++	++	+	±	−	−	−	
BH	−	−	±	+	++	+	−	−	−	−	−	−	
G	−	−	−	+	+	±	−	−	−	−	−	−	Infant
C	−	−	−	+	+	+	±	−	−	−	−	−	strains.
WE	−	−	±	+	+	+	±	±	−	−	−	−	

Monovalent Rabbit Serum Immune to Infant Strain C.

Strain.	Control.	1:20	1:33	1:50	1:66	1:100	1:200	1:333	1:500	1:666	1:800	1:1,000	Infant serum
C	−	±	+	++	+++	+++	+++	+++	+++	+++	+++	++	
BH	−	±	+	++	+++	+++	++	+++	++	+	+	+	Infant
G	−	−	+	++	++	++	+	+	+	±	±	−	strains.
WE	−	−	+	+	++	+++	++	++	++	+	−	−	
Y	−	−	+	+	+	+	±	−	−	−	−	−	
X	−	±	+	++	+	±	−	−	−	−	−	−	Adult
1	−	+	+	+	+	+	+	−	−	−	−	−	strains.
4	−	−	+	++	+	±	±	−	−	−	−	−	

In addition to the experiments given in the tables others were made of a similar nature, but with sera immune to other adult and other infant strains; *e. g.,* Y and A, C and WE. The results were practically the same as with X and BH immune sera. Table IV gives some of the agglutination reactions with serum prepared with Y (adult) and serum prepared with C (infant) strains.

Complement Fixation.—Antigens were made according to several different methods, but the one finally adopted was that given by Park and Williams (6). We found in making these monovalent antigens that it made no difference whether the final heating at 80° C. for one hour was done before or after filtering, or whether the autolysis at 56° C. lasted for one hour or two. Moreover, in the few instances tried there was no difference noted in the same antigen heated at 80° C. for one hour or at 100° C. for twenty minutes. The antigen is used the same day as prepared, if possible, for in some instances it becomes anticomplementary over night. When an antigen is anticomplementary in dilutions commonly used or becomes so in such dilutions as to prevent its being used, refiltering it or reheating it will not remove its anticomplementary character.

The sheep-rabbit hemolytic system was used throughout these experiments, as it was found that the serum of both normal rabbits and those immunized with the gonococcus contained a stronger natural hemolytic amboceptor for hen than for the sheep red corpuscles.

Before performing each experiment a careful titration of the hemolytic system was done, with varying amounts of amboceptor and complement, and for the final test two units of amboceptor and two of complement were used. Moreover, an equally careful titration of the antigen was made and in each case one-half the amount of antigen that was entirely free from anticomplementary action was used. Harrison (7) lays especial stress on the importance of these two initial titrations and our experience confirms his. The tests were all made in the water bath at 37° C.; antigen, immune serum, and complement were incubated for forty-five minutes, then the hemolytic amboceptor and sheep red corpuscles were added, and the total mixture was incubated for one hour. Table V shows the results of antigens made from adult type organisms tested with both adult and infant type immune sera.

TABLE V.

Monovalent Adult Type Antigens Tested for Complement Deviation.

Strain. Dilution of antigen.	Dilution of immune sera.	Serum X (adult).	Serum BH (infant).	Complement.
X (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	Slight hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	No hemolysis	Complete hemolysis	
	0.01 c.c.	Moderate hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
Y (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	Complete hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
A (adult) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	Complete hemolysis	
	0.2 c.c.	Slight hemolysis	Complete hemolysis	
	0.1 c.c.	Slight hemolysis	Complete hemolysis	
	0.07 c.c.	Complete hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
1 (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	Slight hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
2 (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	Slight hemolysis	Complete hemolysis	
	0.07 c.c.	Complete hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

TABLE V.—*Concluded.*

Strain. Dilution of antigen.	Dilution of immune sera.	Serum X (adult).	Serum BH (infant).	Complement.
3 (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Moderate hemolysis	
	0.1 c.c.	No hemolysis	Moderate hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Slight hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
4 (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Slight hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
5 (adult) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

Antigens made from strain 6 were repeatedly anticomplementary in as small amounts as 0.05 c.c.

When monovalent antigens made from the so called adult strains of gonococci were titrated against a monovalent immune serum prepared with an adult strain, complement was bound in dilutions which caused no deviation of the complement when tested with a so called infant type of immune serum. Sera X (adult type) and BH (infant type) were used. Antigens X (adult) and Y (adult) show practically the same results in this test; complement is bound with 0.05 of a cubic centimeter of adult immune serum, but there is no binding whatever with 0.2 of a cubic centimeter of infant immune serum.

This result happens to coincide with the agglutination tests made with these strains and the same adult serum, but it by no means follows that because of a high agglutination titer complement will

be bound with high dilutions of the serum. It has been shown repeatedly that a high content of a serum of one group or set of antibodies does not necessarily imply a high content of others. This is illustrated with strains 1 (adult) and 4 (adult) and immune serum X (adult). With antigen 1 there is only partial binding of complement with 0.07 of a cubic centimeter, while with antigen 4 there was complete binding with 0.05 of a cubic centimeter. Yet the agglutination tests made with these strains and serum show that there was a partial agglutination of strain 1 at a serum dilution of 1:666, while with strain 4 agglutination occurred at a dilution of 1:333 (table II). In every instance complement was bound in the lowest dilutions of the infant immune serum as well as the adult, with the adult antigens, but never with smaller amounts than 0.3 of a cubic centimeter of the infant serum. On the other hand, with these adult antigens complement was always bound with 0.3 of a cubic centimeter of adult immune serum, in every case with 0.1 of a cubic centimeter or less, and in one experiment with 0.03 of a cubic centimeter (antigen X, serum X). Antigens made from strain 6 according to the method used were repeatedly anticomplementary in as high a dilution as 0.05 of a cubic centimeter. An explanation of this is not apparent, but it may be possible to make a usable antigen from this strain by another method; *i. e.,* the freezing and thawing method. Strain 6 was agglutinated with an adult immune serum in a dilution of 1:333 (table II) and with an infant immune serum at 1:100 (table III). Hence it is classed with the adult type of organism. This is borne out by the history of the case which shows that it was isolated from an adult urethritis (table I).

When the infant strain antigens were titrated against the same monovalent infant and adult immune sera (BH and X), similar differences were observed, as shown in table VI.

Complement was bound with 0.03 of a cubic centimeter of BH serum with BH antigen, but there was no binding whatever with this amount of X (adult) serum. In every case complement was bound with 0.1 of a cubic centimeter of infant serum and usually with 0.05 of a cubic centimeter, when these infant strain antigens were used; but there was no binding whatever with this amount of adult immune serum. With infant type antigens complement was bound

TABLE VI.

Monovalent Infant Type Antigens Tested for Complement Deviation.

Strain. Dilution of antigen.	Dilution of immune sera.	Serum BH (infant).	Serum X (adult).	Complemen .
BH (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Slight hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	No hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
G (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	Moderate hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
C (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Complete hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Slight hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
WE (infant) 0.3 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Moderate hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Almost complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
S (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	Slight hemolysis	Complete hemolysis	
	0.07 c.c.	Moderate hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

TABLE VI.—*Concluded.*

Strain. Dilution of antigen.	Dilution of immune sera.	Serum BH (infant).	Serum X (adult).	Complement.
WH (infant) 0.2 c.c. in each tube	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c. in each tube.
	0.3 c.c.	No hemolysis	No hemolysis	
	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	Moderate hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

with somewhat smaller amounts of adult immune serum (averaging 0.2 of a cubic centimeter) than was the case with adult type antigens and infant immune serum (averaging 0.3 of a cubic centimeter). Again it is seen that a high agglutination titer does not imply necessarily a deviation of complement at a high dilution of the serum (strain G).

Antigens made from the three organisms isolated from ophthalmic cases when titrated against monovalent adult serum deviated complement in higher dilutions of this serum than when titrated against infant immune serum (table VII). This difference was well marked with strain CM, but less striking with strains K and L.

It should be emphasized again that in order to determine any degree of specificity whatever of the various strains of gonococci, the smallest amount of complement possible was used (two units) with the largest amount of antigen and immune serum (half the amount that was not anticomplementary). The test so made is an extremely delicate one. It is hardly necessary to add that such quantities would not be practical for the ordinary clinical procedure.

DISCUSSION.

From the agglutination and complement fixation tests just described it is evident that within the large group of organisms known as the gonococcus two more or less distinct types may be differentiated. These types correspond to the clinical source of the strains, whether from an infection of the acute urethritis type in the adult or from the vulvovaginitis of infancy and early childhood. The difference between these types of gonorrheal infection is well recog-

TABLE VII.

Monovalent Ophthalmic Gonococcus Antigens Tested for Complement Deviation.

Strain. Dilution of antigen.	Dilution of immune sera.	Serum X (adult).	Serum BH (infant).	Complement.
K (ophthalmic)	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c.
0.2 c.c.	0.3 c.c.	No hemolysis	No hemolysis	in each tube.
in each tube	0.2 c.c.	No hemolysis	Complete hemolysis	
	0.1 c.c.	Moderate hemolysis	Complete hemolysis	
	0.07 c.c.	Complete hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.02 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
L (ophthalmic)	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c.
0.2 c.c.	0.3 c.c.	No hemolysis	No hemolysis	in each tube.
in each tube	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	No hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	Complete hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	
CM (ophthalmic)	0.4 c.c.	No hemolysis	No hemolysis	0.01 c.c.
0.2 c.c.	0.3 c.c.	No hemolysis	No hemolysis	in each tube.
in each tube	0.2 c.c.	No hemolysis	No hemolysis	
	0.1 c.c.	No hemolysis	No hemolysis	
	0.07 c.c.	No hemolysis	Complete hemolysis	
	0.05 c.c.	No hemolysis	Complete hemolysis	
	0.03 c.c.	Complete hemolysis	Complete hemolysis	
	0.01 c.c.	Complete hemolysis	Complete hemolysis	
	0.007 c.c.	Complete hemolysis	Complete hemolysis	

nized clinically and the immunological tests herewith presented would seem to offer an experimental indication that at least a part of the difference is due to an inherent difference in the two types of organisms causing the infections. It is highly probable that gradations between the two types exist; that the gonococcus may be more or less of a labile group within itself; and that if a large enough number of strains were tested by their immunological reactions certain of these would be found partaking of the characteristics of both types. The conclusion is not to be drawn that the adult and infant types of gonococci form two distinct clear-cut groups separate one from the other, but that certain distinctions exist between them, relative differences which can be brought out by the immunological reactions of agglutination and complement fixation with specific

immune sera. But the distinction between the two types is not an absolute one.

We may add at this point that in no instance did any agglutination of either type of gonococcus occur with sera immune to *Diplococcus intracellularis* or to *Micrococcus catarrhalis*. Moreover, there was no binding of complement with meningococcus antigen and gonococcus immune serum, and none with gonococcus antigens (adult or infant type) with either meningococcus or *catarrhalis* immune serum.

Schwartz and McNeil (4) state in their paper that in the ten cases examined of vulvovaginitis in children under five years of age, the complement fixation test of the patient's blood was negative, although in all the gonococcus was demonstrated in smears and by culture. They thought that the negative results were due to the fact that there was no involvement of the cervix, only the vulva, urethra, and vagina being affected, and that unless the cervix was involved, a positive complement fixation test could not be obtained. In the light of our experiments we feel that the reason the tests were negative may have been because a suitable specific antigen was not used. A polyvalent antigen, it is true, was used, but one made from adult strains only. Therefore, in making an antigen for the clinical complement fixation test for gonococcal infection it would be well to employ strains obtained from infants as well as from adults.

CONCLUSIONS.

1. Two principal types of gonococci may be recognized by suitable immunological tests; *e. g.*, agglutination and complement fixation. These two types correspond to the adult and infant types of infection with the gonococcus, seen clinically.

2. On the basis of the immunological reactions of agglutination and complement fixation the strains of gonococci isolated from three cases of ophthalmia are classed with the adult type.

3. A polyvalent antigen for the serological diagnosis of a gonococcus infection should represent strains of the infant type of organism in order that both types of infection, infant as well as adult, may be recognized.

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STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

VII. The Production of Lobar Pneumonia.*

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In a previous paper the production of pneumonia in rabbits by the Meltzer method of intratracheal inoculation was reported (1). The results by this method have not been constant in the hands of all observers (2). An investigation of the details of the method was undertaken with the hope of determining the causes of this inconsistency.

During the past two years a series of animals has been inoculated intratracheally with pneumococci, but not all the animals developed lobar pneumonia. The only factors which may have varied were the force with which the culture was injected into the respiratory tract and the distance to which the catheter was inserted.

A series of experiments was done to determine the importance of these variables. The force with which the culture was injected was readily controlled. In all cases a Record syringe was used containing five to six cubic centimeters of culture fluid and about fifteen cubic centimeters of air. In the animals in which it was desired to inject the culture with great force, the piston of the syringe was pushed in as rapidly as possible. In those in which it was desired to inject with little force, the piston was pushed in very slowly.

In half the animals the catheter was inserted deeply into a bronchus. In the remainder it was pushed into the trachea just a short way beyond the larynx. The results are recorded in table I.

Discussion of Table I.—From table I it appears that, in order to produce lobar pneumonia in rabbits, it is necessary to introduce the organisms into the deeper portions of the respiratory tract, and probably into the air sacs themselves. This can be accomplished

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TABLE I.

Animal No.	Position of catheter.		Force of injection.		Lesions.	
	Deep in bronchus.	Just beyond larynx.	Great.	Slight.	Lobar pneumonia.	Bronchopneumonia.
231	.....	+	.....	+	.....	.....
232	.....	+	.....	+	.....	+
254	.....	+	.....	+	.....	.....
256	.....	+	.....	+	.....	+
255	.....	+	.....	+	.....	.....
235	.....	+	+	.....	.....	.....
236	.....	+	+	.....	.....	.....
251	.....	+	+	.....	.....	.....
252	.....	+	+	.....	.....	.....
253	.....	+	+	.....	.....	+
229	+	.....	.....	+	.....	+
230	+	.....	.....	+	+	.....
248	+	.....	.....	+	+	.....
249	+	.....	.....	+	+	.....
250	+	.....	.....	+	+	.....
233	+	.....	+	.....	+	.....
234	+	.....	+	.....	+	.....
254*A*	+	.....	+	.....	+	.....
254*B*	+	.....	+	.....	+	.....
246	+	.....	+	.....	+	.....

Summary of Table I.—Table I may be summarized as follows: In the first five animals the culture was gently injected into the trachea just beyond the larynx. At the end of three days the animals seemed normal, but were killed to see what changes were present in the respiratory tract. None of them showed lobar pneumonia, and only two showed a minute patch of bronchopneumonia.

In the second five animals the culture was forcibly injected into the trachea just beyond the larynx. One of these animals died at the end of forty-one hours and showed a small area of consolidation in the right middle lobe. The other four animals were in good condition at the end of three days, when they were killed. In none of them was there any evidence of either lobar or lobular pneumonia.

In the third five animals the culture was gently injected as far down one bronchus as the catheter could be inserted. Two of these animals died in forty-two and forty-five hours, respectively, and both showed lobar consolidation. The other three animals were killed at the end of three days. Two of these had lobar pneumonia while the third had patchy bronchopneumonia.

In the last five animals the culture was forcibly injected as far down one bronchus as the catheter could be inserted. All these animals died in from twenty to forty-six hours. They all showed lobar consolidation.

only by the insertion of the catheter as deeply into the bronchi as possible. Even under these conditions, unless the culture fluid is injected with considerable force, lobar pneumonia will not necessarily result. If the culture is introduced into the upper air pas-

sages with the catheter just beyond the larynx, the lungs are rarely affected.

This suggests that there may be some protective mechanism in the upper respiratory tract whereby the organisms are prevented from reaching the alveoli; and in man, also, the development of pneumonia may be associated with the imperfect functionating of such a mechanism.

During recent years there has been some evidence that pneumonia is a contagion; but while there can be no doubt that this is true at times, it is not apparent in the vast majority of cases. It is generally believed that pneumonia does not develop from infection from without, but that the organisms dwelling upon the mucous membrane of the upper respiratory tract are the cause of the disease. There is no question that if these organisms of the upper respiratory tract reach the alveoli of the lungs in sufficient numbers they will produce pneumonia, just as many other organisms, regardless of their virulence, when experimentally introduced into the alveoli, will produce pneumonia (3).

We have no conclusive evidence in support of the theory that the organisms normally present in the mouth and upper respiratory passages are the cause of pneumonia in man, but, granting this, the terms lowered resistance and predisposition to this disease must mean a disturbance of the protective mechanism of the upper respiratory tract.

This is clearly demonstrated in the experiments noted above, in which animals receiving considerable numbers of pneumococci in the trachea, just beyond the larynx, get rid of them without suffering appreciable involvement of the lungs; whereas those animals in which the same number of similar organisms has been introduced into the air sacs invariably develop lobar pneumonia.

A series of experiments was undertaken to determine the importance of such secondary factors in the etiology of pneumonia, as alcohol, cold, inhalation of ether and bromine, and the control of the vagus upon the upper air passages.

EFFECT OF ALCOHOL AND COLD.

Seven animals were given from 20 to 40 c.c. of 30 per cent. alcohol daily for from one to nine days by stomach tube, according to the method of Frieden-

wald (4). Each day, following the administration of alcohol, the animals were placed in a cool chamber at about 4° C. for a few hours. One of the animals so treated for three days was killed and showed patchy congestion in various lobes. Four others died after they had been given alcohol and exposed to cold. Two of these had intense laryngitis, tracheitis, and bronchitis, and the fourth showed scattered patches of bronchopneumonia. In each of the two remaining animals 1.5 c.c. of a twenty-four hour culture of pneumococci were gently injected into the trachea just beyond the larynx, after the animals had been given alcohol and exposed to cold on nine consecutive days. Death followed in two and three days, respectively. One animal showed uniform congestion of all lobes with intense laryngitis, tracheitis, and bronchitis. The other showed a patch of bronchopneumonia involving about one-twelfth of the left lobe, together with injection of the larynx, trachea, and bronchi.

EFFECT OF ETHER.

Five animals were anesthetized with ether and then 5 c.c. of a twenty-four hour culture of pneumococci in pig serum broth were gently injected into the trachea of each with the catheter inserted just beyond the larynx. The ether anesthesia was continued. Two of the animals died after one half hour's inhalation, and each showed moderate congestion of all lobes. In the remaining three the anesthesia was continued for one hour. At the end of three days the animals were killed. One showed a purulent and fibrinopurulent pleurisy on the right side. The two remaining animals showed patchy consolidation of the left lower lobe.

EFFECT OF BROMINE INHALATION.

Eight animals were subjected to bromine fumes one hour daily for three to ten days. After each exposure the mucous membrane of the nose and mouth was found injected, slightly swollen, and covered with a thin, watery exudate.

In three of these animals 5 c.c. of a culture of pneumococci in pig serum broth were gently injected into the trachea beyond the larynx just before the last bromine exposure. At the end of forty-eight hours the animals were killed. Two showed injection of the larynx, trachea, and bronchi. The third showed acute laryngitis, tracheitis, and bronchitis, and a few small patches of bronchopneumonia in both lungs.

In three of the remaining animals 5 c.c. of a culture of pneumococci in pig serum broth were gently injected into the trachea beyond the larynx a few minutes after the last bromine exposure. These animals were sacrificed at the end of forty-eight hours. One of them showed injection of the trachea; the others showed injection of the larynx and trachea and a few small patches of consolidation in two lobes.

The two remaining animals in the series were given no pneumococci, but were killed forty-eight hours after the last bromine exposure. One showed a diffuse injection of the larynx, trachea, and bronchi; the other showed marked injection of the larynx, trachea, and large and small bronchi, with extensive hemorrhages of the smaller bronchi extending into the adjoining alveoli.

The results of the experiments to determine the influence of alcohol and cold and the inhalation of ether and bromine in the etiology of lobar pneumonia are not conclusive. These factors seem to predispose toward the development of a bronchitis and even bronchopneumonia, both in animals receiving organisms into the upper air passage, and in animals not so treated.

EFFECT OF CUTTING ONE VAGUS NERVE.

In this experiment seven animals were used. In four about one inch of the right vagus nerve (midcervical portion) was resected, and two days later 5 c.c. of a twenty hour culture of pneumococci were gently injected into the trachea just beyond the larynx. At the end of three days the animals were sacrificed. One showed no pulmonary change whatever; one showed a purulent and fibrinopurulent pleurisy and mediastinitis, but no involvement of the lungs; the third had a small patch of consolidation in the right lower lobe; and the fourth showed a patch of consolidation, lobar in type, affecting about one-sixth of the left lower lobe, congestion of the left upper lobe, and bilateral purulent and fibrinopurulent pleurisy.

The fifth animal received 4 c.c. of a twenty-four hour culture of pneumococci in the trachea beyond the larynx eleven and one-half weeks after a right-sided vagotomy. The rabbit was killed seven days later and showed purulent bronchitis, atelectasis of both upper lobes, and extensive fibrinopurulent peritonitis.

The two remaining animals died in three and twenty-two days, respectively, after simple right-sided vagotomy. The first showed early lobar consolidation of the entire right, middle, and left upper lobes, and one-half of the left lower lobe. The other animal showed lobar consolidation of one-half of the right lower lobe and fibrinopurulent pleurisy, mediastinitis, and pericarditis.

B. coli was obtained in pure culture from the consolidated lung of the animal that died three days after vagotomy. In this connection the results of cultures taken from lungs of rabbits developing pneumonia spontaneously are of interest. The disease is not of rare occurrence. A number of the stock animals as well as several dying in the country and brought to the laboratory for investigation showed this lesion. The associated organism was usually one of the intestinal group (*B. coli, B. lactis aerogenes*), although in one instance a pneumococcus was obtained in pure culture.

EFFECT OF CUTTING BOTH VAGI.

Six rabbits were used for this experiment. In five both vagi were resected at the same time. In the sixth the left vagus was cut seven days after a right-sided vagotomy. Although no organisms were injected into the respiratory tract the animals died five to forty-eight hours after both vagi were sectioned. The two animals dying in five hours showed patchy congestion of all lobes. In one there was, in addition, considerable tracheitis and bronchitis. Of the two that died in ten hours, one had patches of congestion in all lobes and both showed bronchitis. The fifth died in sixteen hours and showed intense laryngitis, tra-

cheitis, and bronchitis, bilateral purulent and fibrinopurulent pleurisy, and extensive congestion and early consolidation of all lobes, especially the right lower. The last animal showed pseudolobar consolidation of the entire left upper lobe (innumerable adjoining areas of grayish red consolidation) and congestion and edema of all other lobes. This animal also had marked laryngitis, tracheitis, and bronchitis, and bilateral fibrinopurulent pleurisy. In all but the last animal the esophagus was found engorged with partly digested food and, in one instance, a considerable amount of this material was found in the trachea.

These results are in accord with the findings of previous investigators. There is an abundance of literature upon the relation of the vagus nerve to pneumonia, with various theories of explanation. The controversy of Traube (5) and Schiff (6) (1848–1871) has been followed by the observations and conclusions of many others (Vulpian (7), Piccinino (8), Bettini (9)).

In brief it may be stated that as long ago as 1740 Valsalva (10) noted the occurrence of pneumonia in laboratory animals following double vagotomy.

In 1846 Traube called attention to paralysis of the esophagus as well as the larynx in these animals. Contrary to Schiff, who ascribed the affection to a vasomotor disturbance following the section, Traube attributed the disease to the aspiration into the respiratory tract of saliva and food particles. In support of this theory his demonstration of the ability to produce pneumonia by the injection of saliva and food particles in otherwise untreated animals and the possibility of averting the disease by completely separating the digestive from the respiratory tract is significant.

The above results in animals with both vagi cut reveal the importance of the control of these nerves upon the respiratory tract. When they are both out of function the protective mechanism of the upper respiratory tract is no longer operative, and organisms are allowed to penetrate to the alveoli and there set up an inflammatory process.

CONCLUSIONS.

1. The production of lobar pneumonia in rabbits is dependent upon the introduction of organisms into the alveoli themselves.

2. In order to accomplish this the catheter through which they are injected must be inserted as deeply into a bronchus as possible and the culture fluid injected with considerable force.

3. Large numbers of organisms injected into the trachea just beyond the larynx set up no great changes in the lungs, even though the injection be forcible. This fact suggests the presence of a protective mechanism in the upper air passages, which, under normal conditions, prevents the penetration of organisms into the lungs.

4. If animals be subjected to cold, alcohol, and the inhalation of

irritating gases, the so called secondary factors in the etiology of lobar pneumonia in man, then the injection of pneumococci into the trachea causes inflammatory changes of the upper respiratory tract and occasionally pneumonia.

5. The vagi prevent foreign material in the pharynx and upper respiratory tract from reaching the lungs. Section of one vagus may be followed by pneumonia, while section of both invariably leads to this result.

6. It is possible that the secondary factors mentioned above owe their action to their influence upon the vagus control of the upper respiratory tract.

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STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

VIII. Intra Vitam Staining in Experimental Pneumonia, and the Circulation in the Pneumonic Lung.*

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It has been shown (1) that when pneumonia is produced in a vitally stained animal, the consolidated area is intensely stained, while the remainder of the lung remains very faintly colored. The colorization of the pneumonic area is dependent upon the localization of the dye in the fibrin of the exudate. The nuclei of some of the leucocytes are also stained.

On the basis of these preliminary experiments it was decided to test more carefully the affinity of the dye for the various elements of the exudate at intervals in the progress of pneumonia. In the course of this study phenomena presented themselves which led to a more detailed examination of the circulation in the pneumonic lung.

INTRA VITAM STAINING IN EXPERIMENTAL PNEUMONIA.

The stain used in the experiments was trypan blue. The method of injection was the same as that described in a previous paper (2).

As is well known from the studies of Bouffard (3), Goldmann (4), and others, the tissues of the lung have very little affinity for the dye, and in normal, vitally stained animals only a few of the cells of the interstitial tissue contain blue granules in their protoplasm. Consequently, the lung appears pale pink in contrast to the liver, for example, which is intensely stained.

Schulemann (5) and others have pointed out that the cells of the circulating blood do not absorb the dye, and the only cells that may appear in the blood containing it are huge macrophages similar to those found in the serous cavities of the body. These cells are not found in the pneumonic exudate which is composed of blood cells, chiefly polymorphonuclear leucocytes.

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It has been shown in the unpublished experiments of Evans and Winternitz that the nucleus of a living cell never stains vitally, but that as soon as a cell is injured its nucleus absorbs the stain. In the hands of other observers, Evans and MacCurdy (6), this fact has been successfully used as a guide to the recognition of cell injury when other methods have failed. It was therefore hoped that the fate of the leucocyte in the pneumonic exudate might be determined by this method.

In this experiment forty-one rabbits were used. Some of the animals received one, others two, and still others three intravenous injections of twenty cubic centimeters of a 1 per cent. trypan blue solution. In some cases the dye was given before the intratracheal inoculation of pneumococci, while in others the first dose of trypan blue was given as late as sixty-five hours after the injection of the organisms. The animals were killed at intervals varying from three hours to six and one-half days after the intratracheal injection of pneumococci.

The results of these experiments may be summarized as follows: As early as seven hours after the intratracheal inoculation of pneumococci an occasional polymorphonuclear leucocyte with its nucleus stained intensely blue occurs in the exudate. These apparently dead cells increase in number as the process advances but never form the majority of the cells of the exudate. At first these stained cells have distinctly polymorphous nuclei, but slowly the characteristic shape of the nucleus is lost and it is converted into a small, homogeneous, blue staining mass. Furthermore, as early as three hours after the process has begun, polynuclear cells containing blue granules in their cytoplasm are seen in the alveoli, bronchioles, and blood vessels.

These granules correspond in size, shape, and number to the amphophilic granules of the rabbit leucocytes, and occur in a large number of polynuclear cells at all stages of the disease. As the process advances, in addition to these granules, the cytoplasm occasionally contains within it much larger, irregular, blue masses.

Since vitally stained granules have never been described in polymorphonuclear leucocytes, these findings incited a more careful study of the polynuclear cells in the general circulation of otherwise

untreated, vitally stained animals, and in vitally stained animals in which pneumonia had been produced.

A large number of smears from the ear vein of both these types of animals was studied. The polynuclear cells in these smears were almost constantly unstained; very exceptionally one with blue granules occurred. The significance of this selective staining of the granules of the leucocytes in the pneumonic lung has not been determined.

From the experiments of Winternitz and Evans, it is known that the granules of the polymorphonuclear leucocytes stain with trypan blue when the cell is injured. This injury may be accomplished in many ways; *i. e.*, by mixing the blood with benzol, by pressing the cover-slip forcibly on a fresh blood smear, etc. It is possible, therefore, that the staining of the granules of the leucocytes in the pneumonic lung may result from some injury to the cell membrane. This does not seem probable, since cells are found with only part of their granules vitally stained. The only elements in the pneumonic exudate that stain vitally are these granules and the fibrin. The granules, although at first similar in size and shape to the normal granules of the leucocyte, may become larger and more irregular in shape. It is possible that the staining of these granules may be associated with some functional change within them perhaps related to fibrin formation.

THE CIRCULATION IN THE PNEUMONIC LUNG.

When pneumonia is produced in animals with vital stain in their circulation, the involved lung has a uniform blue color; if, however, the dye is injected sometime after the pneumonia is produced (twenty to sixty-five hours), pale gray consolidated areas occur in the otherwise densely blue stained, involved lobes. Such areas occur most frequently at the periphery of the lobe, but there is no uniformity in their distribution. Similar areas were observed in the pneumonic lungs of animals not vitally stained. Here they appear grayish white and are much less granular on section than the remainder of the consolidated lobe. The sharp demarcation of these wedge-shaped, infarct-like areas was so striking in the vitally stained animals that it led to the following study of the circulation of the lung in pneumonia.

The consensus of opinion concerning the progressive pallor of the pneumonic lung after the stage of engorgement is expressed in the more recent text-books as follows: "The gray color is caused partly by the large number of leucocytes in the exudate and partly by the poor supply of blood in the capillaries which are compressed by the ever increasing exudate". (7). "As the exudation increases in amount and the fibrin meshwork thickens, the air-sacs are greatly distended, the alveolar walls stretched, and the capillaries compressed so that they no longer appear engorged. As a result, the redness due to congestion and to some extent to hemorrhage fades and the lung passes into the stage known as grey hepatization" (8).

The purpose of this investigation was to determine, first, whether there is any impairment of the circulation in the pneumonic lung, and, secondly, if so, how it is brought about.

On Dec. 15, 1913, there came to autopsy a patient with lobar pneumonia involving the right middle and upper lobes. Three and a half hours after the patient's death the lungs were injected through the pulmonary artery, under a pressure of 120 mm. of mercury, with a Berlin blue gelatin solution (equal parts of 5 per cent. Berlin blue and 10 per cent. gelatin solution). After the injection the pulmonary artery was tied and the lungs were placed in 10 per cent. formalin over night. On section the following morning a striking picture presented itself. The consolidated area (right upper and middle lobes) was pale gray, whereas the non-consolidated area (lower lobe) was intensely blue. An extremely small amount of the blue solution had found its way into the vessels of the consolidated area. Four subsequent cases, treated in the same manner, yielded the same result. With the same method, a series of rabbit lungs, showing experimental lobar pneumonia, was injected immediately after the death of the animals and on section these likewise presented the same striking contrast.

Microscopic examination of the human and rabbit lungs entirely corroborated the macroscopic findings. Very little of the blue solution was found in the consolidated areas, whereas the blood vessels of the uninvolved portions were engorged with the blue injection mass.

These experiments prove that after death, under exactly the same conditions, the vascular bed in the consolidated area can be but imperfectly injected with the dye as compared with the uninvolved portions of the same lung. It was next necessary to determine whether this circulatory impairment could be explained by the increased intra-alveolar pressure due to the exudate.

Accordingly a normal rabbit was killed with ether and the lungs were removed. A cannula was inserted into the left bronchus and the left lung was distended with air so that its volume was considerably greater than if it had been the seat of lobar pneumonia. While the left lung was still so distended, an injection mass was forced through the pulmonary artery under a pressure of 120 mm. of mercury. There was apparently no difference macroscopically be-

tween the lobes in which the intra-alveolar pressure was very great and those in which it was slight, either in the rapidity of injection or the amount of dye present.

Microscopic sections also failed to show any difference in the quantity of blue on the two sides.

This experiment was followed by another in which the intra-alveolar pressure was increased by the introduction of a 10 per cent. gelatin solution into the left bronchus under a pressure of 120 mm. of mercury. The solution and the lungs were kept at body temperature during the injection. After the gelatin had been forced into the alveoli the lungs were placed in cold water to allow the gelatin mass to solidify, and, when this had taken place, the lungs were injected through the pulmonary artery with a Berlin blue gelatin mass in the manner above described.

On section gross and microscopic examination failed to reveal any difference between the two sides.

A similar experiment was performed, with freshly drawn rabbit blood to distend the alveoli, and the results of the subsequent vascular injection were identical with those obtained when air and gelatin were used to raise the intra-alveolar pressure.

These experiments prove that the impaired circulation in the pneumonic lung can not be due to the pressure of the exudate within the alveoli. An attempt was then made to determine what element or elements of the inflammatory exudate were responsible for this impairment of the circulation.

A number of rabbits was given benzol until their leucocytes were reduced to below 1,000 per cm., and then in each one 5 c.c. of a culture of pneumococci were injected as deeply into a bronchus as the catheter could be inserted. At the end of forty-eight hours the animals were killed with ether. They all showed lobar type of consolidation. With the same method described above the lungs were injected with Berlin blue gelatin.

On section it was found that the consolidated areas were for the most part grayish white in appearance, whereas the uninvolved lobes were colored deep blue. Microscopically the exudate in the consolidated areas was found to consist almost entirely of fibrin. In addition there was a small amount of serum, an occasional alveolar cell, but very few leucocytes. The vessels in these areas contained very little of the blue injection mass and corresponded in this respect to the vessels of the injected pneumonic lung in normal rabbits.

These experiments indicate that the fibrin is the important element in the interference of the circulation in the pneumonic lung, for pneumonia in aplastic animals is characterized by an exudate of fibrin with an almost total absence of cellular elements (9). An attempt was therefore made to produce pneumonia in animals in

which the fibrin formation had been inhibited. This was accomplished through the use of phosphorus and chloroform.[1]

A rabbit was injected subcutaneously with 0.8 c.c. and 0.4 c.c. of an old solution of phosphorus in olive oil, together with 0.9 c.c. of chloroform in 30 per cent. alcohol intragastrically, on two successive days. Twenty-four hours later the clotting time of the blood was found to be greatly delayed, and the resulting clot very soft and jelly-like. Then 5 c.c. of a twenty-four hour culture of pneumococci in pig serum broth were injected as deeply into a bronchus as the catheter could be inserted. The animal died in seventeen and one half hours, and at autopsy showed consolidation of practically the entire left lower lobe. Berlin blue gelatin was then injected through the pulmonary artery.

On section the consolidated lobe was found to be almost as uniformly blue as the uninvolved lobes. Microscopic section showed that the exudate contained an abundance of cells but very little fibrin. Furthermore, the vessels of the consolidated area contained a much greater amount of dye than had been observed in any of the pneumonic lungs described above.

These experiments demonstrate that the fibrin is responsible for the impairment of the circulation of the lung in pneumonia. A study of the distribution of the fibrin was made to determine how it produced the obstruction.

Microscopic examination of the pneumonic lungs from the above experiments showed a large number of fibrin plugs in the capillaries and even in the larger vessels. The only exception was the consolidated lung of the animal that had received chloroform and phosphorus. The fibrin thrombi have been observed by others (10, 11), but they were considered to be a postmortem, or at most an agonal, phenomenon and of no significance in the pneumonic process.

That they occur early in the course of pneumonia can be demonstrated from the experiment with trypan blue. This is a most readily diffusible dye and reaches all areas whose blood supply is not impaired. When pneumonia is produced in animals in whose circulation this dye is present, the consolidated area becomes uniformly stained. If the dye is injected twenty hours after the pneumonia is produced, the consolidated area is not uniformly stained, and, as has been said, it may show sharply demarcated and absolutely unstained areas.

It may, therefore, be concluded that fibrin plugs form in the capillaries early in pneumonia and that these impair the circulation in the consolidated lung. The distribution of the fibrin plugs in the ves-

[1] We are indebted to Drs. Whipple and Goodpasture for aid in this experiment.

sels in the pneumonic lung of man is relatively uniform throughout, and by their interference with the circulation they probably cause the pallor of the lung in gray hepatization. In the pneumonic lung of the rabbit the distribution of these plugs is not uniform; they may be so extensive that there is an absolute loss of circulation in local areas. Such areas are likely to undergo necrotization and probably result in the minute abscesses which have been found repeatedly in rabbits' lungs long after the pneumonic process has subsided.

The importance of this wide-spread formation of fibrin plugs in the vessels in the pneumonic lung is at once evident. The experiments of Opie (12) have shown the presence of an antitryptic substance in the serum which prevents the autolysis of leucocytes and the digestion of fibrin by leucocytes. The leucocytes alone digest this material rapidly. Therefore, with an interference of the circulation the leucocytes of the exudate are much less influenced by the action of the serum which reaches them in minimum amount, and as a result autolysis takes place in the exudate just as it would if the pneumonic lung were placed in the thermostat. There can be no doubt that this interference in the circulation in the pneumonic lung is of great importance in the resolution of the exudate.

It is further probable that the interference of the circulation in the pneumonic lung is responsible for the restricted action of immune serum in this disease. The experiments of Cole (13) have shown that the septicemia of pneumonia, and consequently the fatal outcome of the disease, may be averted in many cases by the intravenous administration of the specific immune serum, but the progress of the local lesion remains uninfluenced. This must be expected since, on account of the impaired circulation, the immune serum can not reach the local lesion in sufficient amounts to influence its progress. It is evident that this same criticism would apply to any therapeutic measure so administered, and it therefore seemed advisable to ascertain whether the consolidated area could be impregnated in some other way.

The following experiments were carried out to see whether the consolidated area could be stained by an intratracheal injection of a dye.

Pneumonia was produced in a rabbit, and forty-eight hours later, under ether anesthesia, the anterior thorax was removed, and artificial respiration by the Meltzer method of intratracheal insufflation was instituted. Respiration was interrupted long enough to allow the injection of 10 c.c. of a 1 per cent. solution of trypan blue into the consolidated lung through a catheter inserted into its bronchus. The animal was then killed by ether.

On section of the consolidated lung it was found to be uniformly and intensely stained. Microscopic section showed the presence of the dye in practically all the alveoli localized in the same elements which are stained after intravenous injection of the dye.

This experiment was repeated with a similar result. It may be said, therefore, that the presence of fibrin plugs throughout the capillary bed of the pneumonic lung interferes greatly with the penetration even of such a diffusible substance as trypan blue, when this drug is injected intravenously, but the exudate offers no serious obstruction to the penetration of the dye into the alveoli when it is injected intrabronchially.

The method of intrabronchial treatment of pneumonia therefore suggests itself.

CONCLUSIONS.

1. Dead leucocytes are constantly found in the pneumonic exudate. They rapidly undergo disintegration. Up to the seventh day they do not form the majority of the cells of the exudate.

2. Polymorphonuclear leucocytes with vitally stained granules are present in the exudate, vessels, and interstitial tissue of the lung in experimental pneumonia, but they are not demonstrable in the general circulation in the same animals.

3. There is a marked impairment of the circulation in the pneumonic lung.

4. The increase in the intra-alveolar pressure exerted by the exudate has no influence upon the circulation.

5. The impaired circulation results from the wide distribution of capillary fibrin thrombi. In man these are, as a rule, distributed with relative uniformity. In the rabbit this is not usually the case. The thrombi are much more abundant in some areas and may lead to localized necrosis.

6. The impairment of the circulation is of importance in bringing about resolution. Only enough blood is allowed to seep through

the vessels to nourish the alveolar walls. Consequently very little serum escapes into the alveoli and the autolysis of the exudate by the leucocytes is unhindered.

7. The impairment of the circulation in the pneumonic lung seriously interferes with the action of intravenous therapy upon the local lesion.

8. The exudate in the pneumonic lung can be readily impregnated with a dye injected intrabronchially. This suggests a method of administration of therapeutic agents in pneumonia.

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STUDIES UPON EXPERIMENTAL PNEUMONIA IN RABBITS.

IX. The Part of the Leucocyte in the Immunity Reaction.*

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It has been shown that pneumonia is much more fatal in aplastic than in normal rabbits and that the latter are less resistant to the disease than animals in which a leucocytosis has been artificially produced (1). Recently Gay (2) has reported a hyperleucocytosis following the intravenous injection of antigen in immunized animals, thus indicating the importance of the leucocyte in the immunity reaction as well as in the initial defense. It was therefore determined to study the part of the leucocyte in the immunity reaction.

In the following paper the effect of the intravenous injection of pneumococci in normal, aplastic, and in actively and passively immunized normal and aplastic rabbits will be presented.

METHOD.

It is possible to follow the extent of the bacteriemia in pneumonia by determining the number of organisms in relation to the number of red blood cells. This method is not satisfactory when the organisms are present in small numbers; then they can not be counted accurately or are entirely overlooked. Therefore the following method of making blood cultures was devised. The animals' ears were shaved and the skin was sterilized with 95 per cent. alcohol. One of the larger veins was then punctured with a sterile needle, and a loop of blood was immediately smeared over the surface of a rabbit blood agar slant. This method allows of rapidly repeated cultures and does not interfere with the condition of the animal.

All the cultures in the experiments to be reported were made in the manner described. In all the same amount of blood was taken. The tubes were incubated for forty-eight hours before observations were recorded.

* Received for publication, January 23, 1915.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN NORMAL ANIMALS.

Fourteen normal rabbits were given an overwhelming dose of pneumococci intravenously. Blood cultures and smears were made at short intervals until the animals died. In several a number of leucocyte counts were made. The results were similar in all cases (table I).

TABLE I.

Date (1914).	Time.	Procedure.	No. of pneumococci in blood smears.	No. of colonies in blood cultures.	White blood count per c.mm.	Result.
June 3	9 A.M.	.....	.....	.....	11,700	.....
	10 A.M.	8 c.c. pneumococci intravenously	.....	.....	.....	.....
	10.10 A.M.	.....	Too few to count	Innumerable	.....	.....
	11 A.M.	.....	Too few to count	Innumerable	.....	.....
	3 P.M.	.....	400,000 per c. mm.	Innumerable	.....	.....
	5 P.M.	.....	800,000 per c. mm.	Innumerable	.....	.....
	7 P.M.	.....	1,335,000 per c. mm.	Innumerable	2,200	.....
	9 P.M.	.....	3,800,000 per c. mm.	.....	3,900	.....
June 4	1 A.M.	.....	.....	.....	4,400	.....
	10 A.M.	.....	.....	.....	.....	Death.

It may be seen from table I that following an intravenous injection of a lethal dose of pneumococci in a normal rabbit the organisms increase steadily in number until the animal dies. Further, there is a marked and persistent fall in the number of leucocytes in the peripheral circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN APLASTIC ANIMALS.

Experiments with several rabbits in which the leucocytes had been reduced to below 1,000 per cubic millimeter by the daily injection of benzol showed that these animals are more susceptible than normal rabbits to the intravenous injection of pneumococci. No special experiments were undertaken to determine the leucocytic reaction, as the result of such a procedure seemed sufficiently evident from previous experiments in which pneumococci had been injected intratracheally in aplastic animals. Here a bacteriemia developed while the number of leucocytes remained uninfluenced.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN PASSIVELY IMMUNIZED RABBITS.

Three rabbits were passively immunized and then injected intravenously with an overwhelming dose of homologous pneumococci. Blood cultures were taken at short intervals over a period of twenty-seven to seventy-two hours. The results in all were practically identical (table II).

TABLE II.

Date (1914).	Time.	Procedure.	No. of colonies in blood cultures.	Result.
June 8	9 A.M.	20 c.c. immune serum	.....	.....
	10 A.M.	10 c.c. virulent pneumococci intravenously	.....	.....
	10.05 A.M.	.....	Very many	.....
	10.30 A.M.	.....	None	.....
	11 A.M.	.....	None	.....
	2 P.M.	.....	None	.....
	8.30 P.M.	.....	None	.....
June 9	1 P.M.	.....	None	Recovery.

Table II shows that, following the intravenous injection of a lethal dose of pneumococci in a passively immunized rabbit, the organisms are present in large numbers for the first five minutes, but are no longer demonstrable in the circulation thirty minutes after the injection.[1]

[1] In the other two rabbits in this series the time of disappearance of the organisms was one hour and three hours, respectively. All three animals recovered completely.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN ACTIVELY IMMUNIZED RABBITS.

In this experiment fifteen rabbits were used. The animals received a number of gradually increasing doses of pneumococci until they possessed a decided immunity. They were then injected intravenously with a lethal dose of pneumococci of the same strain. Blood cultures were taken and the leucocytes counted at short intervals after the injection. The chart of animal 2 (table III) is characteristic of this group.

TABLE III.

Date (1914).	Time.	Procedure.	No. of colonies in blood culture.	White blood count per c.mm.	Result.
Apr. 17 to May 23	.....	Gradually increasing doses of pneumococci intravenously from 0.5 c.c. to 9.0 c.c.	.....	.....	.....
June 3	9.15 A.M.	.....	.....	10,800	.....
	10.15 A.M.	8 c.c. of pneumococci intravenously	.....	.....	.....
	10.20 A.M.	.....	Innumerable	.....	.....
	10.30 A.M.	.....	Very many	.....	.....
	10.45 A.M.	.....	Many	.....	.....
	11.15 A.M.	.....	50	.....	.....
	12.15 P.M.	.....	12	.....	.....
	2.15 P.M.	.....	None	.....	.....
	4.15 P.M.	.....	None	.....	.....
	7.15 P.M.	.....	None	23,300	.....
	10.15 P.M.	.....	None	37,300	.....
June 4	1.15 A.M.	.....	None	27,900	.....
	10.15 A.M.	.....	None	.....	Recovery.

Table III shows that in actively immunized rabbits the injection of a lethal dose of pneumococci is followed by a rapid disappearance of the organisms from the circulation, and that there is a decided leucocytosis reaching its maximum about twelve to fifteen hours after injection. This leucocytosis occurs many hours after the disappearance of the organisms from the circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN APLASTIC PASSIVELY IMMUNIZED RABBITS.

Eight rabbits were given daily doses of benzol subcutaneously until the number of their leucocytes was below 1,000 per c.mm. At this stage they were passively immunized and then injected with a lethal dose of the homologous pneumococci. Blood cultures were taken at short intervals, and in one animal leucocyte counts were made before and after the injection of the organisms. All these animals reacted identically (table IV).

TABLE IV.

Date (1914).	Time.	Procedure.	No. of colonies in blood cultures.	White blood count per c.mm.	Result.
May 27 to June 2	.....	Daily subcutaneous injections of benzol	.....	.....	.....
June 3	8.30 A.M.	.....	.....	340	.....
	8.45 A.M.	10 c.c. immune serum intravenously	.....	.....	.....
	9.30 A.M.	10 c.c. immune serum intravenously	.....	.....	.....
	9.45 A.M.	8 c.c. pneumococci intravenously	.....	.....	.....
	9.50 A.M.	.....	Many	.....	.....
	10.15 A.M.	.....	36	.....	.....
	10.45 A.M.	.....	6	.....	.....
	11.45 A.M.	.....	1	.....	.....
	1.45 P.M.	.....	1	.....	.....
	3.15 P.M.	.....	6	160	.....
	7.45 P.M.	.....	None	80	.....
June 4	9.45 A.M.	.....	Very many	.....	.....
June 5	3.45 A.M.	.....	Innumerable	.....	Death.

Table IV shows that after the intravenous injection of a lethal dose of pneumococci in passively immunized aplastic rabbits there is a rapid disappearance of the organisms from the circulation; within two to four hours they have practically disappeared. The disappearance of the organisms from the blood is, however, only temporary. The bacteriemia recurs and there is a steady increase in the number of organisms until the animal dies. In addition, it may be noted that the injection of the pneumococci in these animals is not followed at any time by an increase in the number of leucocytes in the peripheral circulation.

THE FATE OF INTRAVENOUSLY INJECTED PNEUMOCOCCI AND ITS RELATION TO THE LEUCOCYTIC REACTION IN ACTIVELY IMMUNIZED APLASTIC RABBITS.

Three animals were actively immunized in the same manner as those previously mentioned. When they possessed a decided immunity they were given daily subcutaneous injections of benzol until their leucocytes were reduced to below 1,000 per c.mm. At this stage they were each injected intravenously with a lethal dose of pneumococci of the same strain used before, and blood cultures were taken at short intervals thereafter. In these animals leucocyte counts were also made several times after the injection of the organisms.

The reaction in all cases was the same and is illustrated by table V.

TABLE V.

Date (1914).	Time.	Procedure.	No. of colonies in blood cultures.	White blood count per c.mm.	Result.
Apr. 17 to May 23	.....	Gradually increasing doses of pneumococci injected intravenously up to 9 c.c.	.....	.....	.....
May 25 to June 2	.....	Daily subcutaneous injections of benzol	.....	.....	.....
June 3	8.50 A.M.	8 c.c. pneumococci intravenously	.....	.....	.....
	9.00 A.M.	.....	Innumerable	280	.....
	9.20 A.M.	.....	Innumerable	.....	.....
	9.50 A.M.	.....	36	.....	.....
	10.20 A.M.	.....	3	.....	.....
	11.20 A.M.	.....	1	.....	.....
	1.20 P.M.	.....	None	.....	.....
	7.20 P.M.	.....	None	380	.....
	9.20 P.M.	.....	None	560	.....
June 4	12.20 A.M.	.....	None	500	.....
	9.20 A.M.	.....	None	380	.....
June 5	3.20 A.M.	.....	None	580	Recovery.

Table V shows that, following the intravenous injection of a lethal dose of pneumococci in actively immunized aplastic animals, there is a permanent disappearance of the organisms from the circulation, and the rabbit recovers.

In these actively immunized aplastic animals there is no increase in the number of leucocytes at any time after the injection of the pneumococci.

SUMMARY AND DISCUSSION.

Following the intravenous injection of an overwhelming dose of pneumococci in normal animals there is a rapidly increasing bacteriemia which reaches its maximum with the death of the animal.

Immunized animals, whether the immunity is active or passive, whether the animals have their leucocytes, or whether these have been destroyed by benzol, react differently. They have in common the ability to cause the organisms to decrease rapidly in number, and, as far as could be determined by the methods used, to disappear absolutely in a very short time from the circulation. This may be called the immediate reaction.

In actively and passively immunized normal rabbits the disappearance of the organisms from the blood is followed by their destruction in the body and the ultimate recovery of the animal. This same result occurs also in actively immunized rabbits deprived of their polymorphonuclear leucocytes by benzol. On the contrary, in passively immunized rabbits, deprived of their leucocytes in the same way, the immediate disappearance of the organisms from the circulation is followed, after a lapse of from six to twenty-four hours, by a recurrent, gradually increasing bacteriemia and the death of the animal.

It is evident that the immediate reaction is no index of the ultimate result.

This finding, that the result of the intravenous injection of an overwhelming dose of pneumococci in immunized animals may be divided into two stages, immediate and ultimate, has been utilized in the interpretation of the experiments that have been reported above.

1. *Normal Rabbits.*—In the normal rabbit the injection of a lethal dose of pneumococci is followed by the same immediate and ultimate response. The animal develops a rapidly progressing bacteriemia and dies.

2. *Passively Immunized Normal Rabbits.*—A diametrically opposed finding is obtained when the animals are passively immunized. Then following the intravenous injection of pneumococci the organisms rapidly disappear from the circulating blood and the animal recovers. The only apparent difference between these two groups of experiments is the introduction of a relatively small quantity of immune serum. Tentatively, therefore, it may be concluded that the immune serum is responsible both for the immediate and ultimate reaction in this case.

3. *Passively Immunized Aplastic Rabbits.*—A normal rabbit that has been previously benzolized, and in this way deprived of its myeloid elements, can not be successfully immunized. Here the immune serum injected even in excessive quantities is followed by the immediate reaction, but the ultimate result is entirely different, —the animal dies.

It may be concluded, therefore, that the serum is the potent factor in bringing about the immediate disappearance of the organisms from the circulation.

Furthermore, it is evident that the white blood cells of the myeloid tissue are necessary in order that passively immunized animals may recover following the introduction of a lethal dose of pneumococci. So far it seems that two elements are essential in the immunity process; *i. e.,* immune bodies and white blood cells.

Corroborative evidence of the importance of the white blood cell in this reaction is offered by the hyperleucocytosis which follows the injection of antigen in actively immunized animals (Gay). This hyperleucocytosis occurs in actively immunized rabbits injected with pneumococci, after the organisms have disappeared from the circulation. It occurs at about the same time that the septicemia recurs in passively immunized aplastic rabbits.

4. *Actively Immunized Aplastic Rabbits.*—The experiments with actively immunized aplastic rabbits complicate the conception of the part of the white blood cells in the immunity process. When actively immunized, benzolized animals are injected with a lethal dose of pneumococci, the immediate reaction occurs just as in the passively immunized aplastic animal. Available antibodies are present and cause the disappearance of the organisms from the circulation. The ultimate reaction differs from that in the passively immunized aplastic animal. The latter develops a recurrent bacteriemia and dies. The actively immunized aplastic animal recovers.

It would seem that the white blood cell is no longer necessary in an animal that has been actively immunized, but that it must be present for the passive protection of the animal.

The ultimate reaction in immunized rabbits seems to be dependent upon some action of the white blood cells. In the passively immunized animal this may occur at the time of, or following, the im-

mediate reaction; and if it can not occur, owing to the absence of the white blood cells, the animal subsequently dies. In the actively immunized aplastic rabbit this action has apparently occurred at a previous time and is sufficiently developed to protect the animal even though the leucocytes have been destroyed.

It may be assumed that the function of the white blood cell is exercised not directly, but perhaps indirectly, by some influence exerted on other body cells. This interrelation or interaction between the white blood cells and other cells in the body constitutes a third factor essential to the ultimate protection of the animal.

Additional evidence of this action of the white blood cell is furnished by further experiments with actively immunized aplastic rabbits. Several immunized rabbits were benzolized and then injected with a lethal dose of pneumococci. The results were the same as those indicated above. The organisms disappeared rapidly from the circulation and the animals recovered.

An interval of five days was allowed to elapse and the animals were injected again with the same amount of pneumococci. This time the reaction was different. The organisms disappeared rapidly from the circulation, but there was a recurrent bacteriemia and the animals died. In these animals the favorable ultimate result after the first inoculation of a lethal dose of pneumococci probably depended upon the presence of the immune bodies and the third factor mentioned above. After the subsequent inoculation of pneumococci the immediate reaction was followed by the recurrence of the bacteriemia and the death of the animals. It may, therefore, be inferred that the third factor present at the time of the first injection had been destroyed, and, owing to the absence of white blood cells at the time of the subsequent inoculation, it was not formed again.

It may be concluded that there are at least three elements necessary in the immunization process: (1) immune bodies, (2) white blood cells, and (3) a third factor which is dependent for its existence upon the presence of white blood cells at the time of inoculation of the pneumococci. Furthermore, this third factor may be removed if the animal is inoculated at a time when it is aplastic.

CONCLUSIONS.

1. The result of the intravenous injection of pneumococci in immunized rabbits may be divided into two stages,—immediate and ultimate.

2. The immediate reaction is not decisive of the ultimate result.

3. The immunity process seems to be dependent upon at least three factors: immune bodies, white blood cells, and a third factor which is dependent for its existence upon the presence of the white blood cells at the time of the inoculation of the pneumococci.

This third factor may be removed by rendering an immunized normal rabbit aplastic and then injecting it with a minimal lethal dose of pneumococci.

The result of the injection of this antigen into immune rabbits varies according to the presence of the three factors mentioned above. The immune bodies cause an immediate disappearance of the organisms from the circulation. The third factor causes the permanent absence of the organisms from the circulation and the recovery of the animal. The white blood cells seem to be essential for the production of this third factor.

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THE INFLUENCE OF EPINEPHRIN UPON THE CORONARY CIRCULATION OF THE MONKEY.*

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PLATE 22.

Epinephrin (adrenalin) undoubtedly dilates the coronary arteries of the animals that are usually made the objects of laboratory investigation. That a better blood supply should thus be afforded the heart by a hormone which arouses a marked increase in activity seems a suitable provision of nature.

The most important ways in which epinephrin may influence the coronary circulation probably are: (1) directly, by acting upon the myoneural junctions of the vessels themselves; (2) indirectly, (a) by changes in the activity of the heart, which may affect the vessels by the production of metabolites (Markwalder and Starling[1]), or mechanically, (b) by changes in the general arterial pressure. (c) by possible actions on the central nervous system.

As has been shown by the classical studies of Langley and his coworkers, the direct action of epinephrin upon the coronary vessels depends presumably upon their innervation. In the laboratory animals thus far investigated these arteries appear to be innervated by dilators of true (thoracicolumbar) sympathetic origin, because epinephrin increases the coronary flow in perfused isolated hearts (whether active or at rest) and causes a relaxation in isolated coronary strips or rings.

Electrical stimulation of the accelerator nerve was found by Maass[2] to increase the coronary flow in the cat, thus indicating in a more direct manner the existence of coronary dilators of sympathetic

* Part of the expenses of this work were defrayed from the Francis E. Loomis Research Fund of the Yale University School of Medicine. Received for publication, January 30, 1915.

[1] Markwalder, J., and Starling, E. H., *Jour. Physiol.*, 1913, xlvii, 275.

[2] Maass, P., *Arch. f. d. ges. Physiol.*, 1899, lxxiv, 281.

origin in this animal. This has recently been corroborated by Morawitz and Zahn.[3]

These authors were impressed by the fact that for the study of many questions relating to the coronary circulation the intact animal is best adapted. The indirect factors in the living animal, of course, modify the influence of the direct. This had been recognized previously by Bond,[4] who worked on the coronary circulation of intact animals in 1910.

Morawitz and Zahn inserted a catheter through the right auricle into the coronary sinus of the hirudinized cat or dog. After measuring with a suitable flow recorder the coronary blood thus collected, they returned it through a slow feeding burette into the jugular vein. Records were obtained of the increase in coronary flow resulting from raising the general arterial pressure in various ways.

The effect of epinephrin upon the coronary flow was then shown to be one of extreme augmentation, even out of proportion to the increase in general arterial pressure. This indicated again the influence of other factors such as increased metabolites or an actively stimulated vasodilator mechanism. Similar, although less marked, effects were obtained by subcutaneous injections of epinephrin.

Angina pectoris was then treated in this manner by Büdingen[5] at the instigation of Morawitz and Zahn. No positive results being obtained, these authors were led to remark that it is by no means clearly established that anginal attacks are due to coronary spasm.

On the other hand, one of us[6] obtained evidence three years ago that the action of epinephrin in man differs from its action in the usual laboratory animals as regards the coronary arteries. It was demonstrated that isolated rings of these arteries obtained from fresh cadavers respond to epinephrin by contraction only. These experiments were controlled by many upon coronary rings of the calf, sheep, and pig, in which was constantly seen the usual relaxation. The conclusion was drawn that epinephrin constricts the

[3] Morawitz, P., and Zahn, A., *Zentralbl. f. Physiol.*, 1913, xxvi, 465; *Deutsch. Arch. f. klin. Med.*, 1914, cxvi, 364.

[4] Bond, G. S., *Jour. Exper. Med.*, 1910, xii, 575.

[5] Büdingen's results are reported by Morawitz and Zahn, *Deutsch. Arch. f. klin. Med., loc. cit.*, p. 388.

[6] Barbour, H. G., *Jour. Exper. Med.*, 1912, xv, 404.

human coronary vessels and that they are therefore supplied, presumably, with vasoconstrictors of true sympathetic origin.

Morawitz and Zahn express themselves as willing to entertain this view as an alternative explanation of Büdingen's negative results with epinephrin in angina pectoris.

The constricting influence of epinephrin upon the human coronaries has, however, been doubted by some orthodox students of epinephrin; for example, Park.[7] With the hope of obtaining corroborative evidence from the monkey the following work was undertaken.

METHOD.

The experiments were made upon normal members of the species *Macacus rhesus,* the control animals being normal rabbits. In these two classes of animals we sought a variation in the action of epinephrin upon the coronary vessels themselves. We selected, therefore, a method which excluded some of the above mentioned indirect factors, employing isolated hearts. For this purpose the perfusion apparatus described by Locke and Rosenheim[8] was found well adapted.

The animals were decapitated and the blood was collected into a vessel containing 0.02 to 0.04 of a gram of hirudin dissolved in 50 cubic centimeters of Locke solution. The blood mixture was filtered and diluted further when necessary to make about 150 cubic centimeters. The blood constituted one-third to one-half of the total mixture.

The heart was immediately excised and connected with the aortic cannula of the perfusing system. After thorough irrigation of the coronary vessels with Locke solution, the blood mixture was transferred to the reservoir of the apparatus and the experiment begun.

In order to obtain a uniform record of the coronary flow it was found advisable to suspend the heart, apex upward, thus preventing the accumulation of blood in the right auricle and ventricle, which leads to an irregular outflow from the beating heart. The heart was held in this position, below, by means of a fixed, curved aortic cannula and above by a thread sutured to the apex of the ventricles.

The rate of coronary flow was measured with Condon's[9] tipper

[7] Park, E. A., *Jour. Exper. Med.*, 1912, xvi, 532.

[8] Locke, F. S., and Rosenheim, O., *Jour. Physiol.*, 1907–8, xxxvi, 205.

[9] Condon, N. E., *Jour. Physiol.*, 1913, xlvi, p. xlvi.

recorder. The perfusion fluid unit was constant during each experiment. It varied somewhat in the series, averaging about 2 cubic centimeters. The injections were made obliquely through the rubber tubing at a point a few centimeters above the aortic cannula. To avoid changes in arterial pressure this was done slowly and with the needle directed against the current of the perfusing fluid.

As shown in the tables, perfusion pressures of 50, 75, and 100 millimeters of mercury were employed. The perfusion fluid was maintained at a constant temperature of 38° C.

Commercial adrenalin chloride was used throughout the work.

RESULTS.

The experiments are summarized in tables I and II. In these it will be noted that no time has been allowed between the injection and the arrival of epinephrin at the coronary vessels. Thus the degree of response is in all cases underestimated. A further source of underestimation in the tables is the fact that the three minute interval chosen often expired before the full effect of the drug was seen. It suffices, however, for demonstrating the main fact, which is a qualitative one.

A better conception of the character of the individual results is obtained from the reproduced tracings. In these the upper line records the rate of coronary flow, the lower the time in five second intervals.

Rabbit Controls.—Seven experiments were made upon two rabbit hearts. The first table shows that all doses employed, varying from 0.025 to 0.25 of a milligram, gave constantly an increase in coronary flow.

TABLE I.

Isolated Rabbit Hearts.

Animal.	Experiment.	Perfusion pressure.	Epinephrin.	Units of perfusion fluid in 3 min.	
				Before epinephrin.	After epinephrin.
Rabbit 1 (3,300 gm.).....	1	50 mm. Hg.	0.00025 gm.	3.2	5.4
	2	50 mm. Hg.	0.0001 gm.	3.0	3.8
	3	50 mm. Hg.	0.00025 gm.	1.8	2.9
Rabbit 2 (1,600 gm.).....	4	100 mm. Hg.	0.0001 gm.	7.2	13.3
	5	100 mm. Hg.	0.000025 gm.	3.2	3.8
	6	100 mm. Hg.	0.0001 gm.	3.1	4.3
	7	50 mm. Hg.	0.0001 gm.	1.5	3.2

Figure 1 is a sample record from a rabbit's coronary flow (experiment 1). A pre-injection period is shown, followed by the increase produced by epinephrin and a partial return to normal.

Monkeys.—That constriction of the coronaries by epinephrin is as constant in the isolated monkey heart as dilatation of the coronaries in the rabbit heart is shown by table II.

TABLE II.

Isolated Monkey Hearts.

Animal.	Experiment.	Perfusion pressure.	Epinephrin.	Units of perfusion fluid in 3 min.	
				Before epinephrin.	After epinephrin.
Monkey 1, ♂ (1,400 gm.)..	8	50 mm. Hg.	0.00025 gm.	9.3	7.8
	9	50 mm. Hg.	0.00025 gm.	6.0	5.2
Monkey 2, ♀ (1,200 gm.)..	10	75 mm. Hg.	0.0005 gm.	13.4	13.1
	11	50 mm. Hg.	0.0005 gm.	5.8	5.4
	12	50 mm. Hg.	0.001 gm.	4.9	4.5
	13	50 mm. Hg.	0.002 gm.	4.2	3.5
	14	50 mm. Hg.	0.002 gm.	10.0	3.6
	15	75 mm. Hg.	0.002 gm.	3.0	1.8
Monkey 3, ♂ (1,800 gm.)..	16	100 mm. Hg.	0.000025 gm.	5.8	4.7
	17	100 mm. Hg.	0.0001 gm.	3.7	3.2
	18	100 mm. Hg.	0.0001 gm.	1.8	1.4
	19	100 mm. Hg.	0.001 gm.	4.2	3.8
	20	100 mm. Hg.	0.0001 gm.	8.2	6.3
	21	100 mm. Hg.	0.002 gm.	6.7	4.3

Fourteen experiments were made upon the hearts of three monkeys, the doses varying from 0.025 to 2 milligrams.

Figure 2 (experiment 8) is from the coronary flow of a male monkey, weighing 1,400 grams, a few minutes after removal of the heart from the body. The beat was active and regular; the heart rate per ten seconds is indicated by the numbers written on the tracing between the flow and time records. By comparison of the eight minute periods before and after the injection of 0.00025 of a gram of epinephrin, it will be seen that 26 perfusion units before were succeeded by 21 units after. The heart rate increased from 84 to 108 as a result of the injection. The tracing shows also the beginning of recovery from the coronary constriction.

Figure 3 illustrates the coronary flow of a female monkey, weighing 1,200 grams, several hours after removal of the heart, being the last of the series on the animal (experiment 14). Two milligrams of

epinephrin were given. The heart had ceased to beat for some time, but had been active during the earlier experiments tabulated from this animal.

The third monkey was a male weighing 1,800 grams. Experiment 17, from this monkey, is illustrated by figure 4. The heart was beating rhythmically before this experiment but within a minute after the epinephrin injection went into partial block, from which it never recovered. After a number of further injections with epinephrin had all yielded constriction, 0.025 of a cubic centimeter of amyl nitrite was injected. The marked flow increase illustrated in figure 5 (experiment 22) was the result. This indicates that the coronaries of the monkey, as of other animals, respond to amyl nitrite by dilatation.

DISCUSSION.

Our results greatly enhance the value of Barbour's earlier experiments upon isolated human coronaries, by annulling all the objections to the theory of coronary constriction by epinephrin in man, which are based upon analogy with other classes of mammalia. There exists, we believe, between certain primates, on the one hand, and certain of the lower mammalia on the other, a previously unsuspected difference in innervation. It has been detected by means of an agent which is already known to exhibit variations in its action upon the uterus of different species. Examples of this, with the literature, will be found in a recent paper by Gunn.[10]

As regards man, the constricting influence of epinephrin upon the coronaries, while having a possible bearing upon the therapy of anginal attacks, may also prove to be related to their etiology. In view of the work of Cannon[11] and others upon the discharge of epinephrin into the blood stream in certain emotional states, an explanation may be found for the frequent association of anginal attacks with excitement.

A factor which is common to all species and which operates against coronary constriction has been purposely omitted from the present work; namely, the decided increase in arterial pressure.

[10] Gunn, J. A., and Gunn, J. W. C., *Jour. Pharmacol. and Exper. Therap.*, 1914, v, 527.

[11] Cannon, W. B., and de la Paz, D., *Am. Jour. Physiol.*, 1911, xxviii, 64.

With stationary perfusion pressures of 50, 75, or 100 millimeters of mercury, which were never changed during an epinephrin experiment, no essential differences were seen in the results. When the pressure was raised or lowered between two experiments a corresponding increase or diminution in coronary flow was, of course, noted. Central effects of epinephrin, which are presumably of minor importance, have also been excluded from this work.

The factor of heart activity is important and has been dealt with to some extent. The earlier experiments upon each animal were all made upon actively beating hearts; many of the later ones upon hearts which were quiescent and remained so. We can say with certainty that the action of epinephrin upon the coronary flow was constant under both sets of conditions. We gained further the impression that the constrictor effect was more marked in resting than in active hearts, which may have been due to the lack of the nonvolatile metabolites referred to by Starling and Markwalder. The variation in dosage in the present work precludes decision in this matter.

The dose was varied in the monkey from 0.025 of a milligram, which was ineffective in one experiment (not tabulated), up to two milligrams. Qualitatively our results were constant. The data are not sufficient to warrant quantitative deductions.

CONCLUSIONS.

Decrease in coronary flow was the constant response of freshly isolated monkey hearts to epinephrin. These hearts were perfused with autogenous hirudinized blood diluted with Locke solution. The results were constant at high or low perfusion pressures, in beating or resting hearts, and with all adequate doses. Increased coronary flow was obtained constantly in rabbit hearts under identical conditions.

In the light of previous work upon isolated human coronary arteries, the general conclusion is drawn that, while actively dilating the coronary vessels in the dog, cat, rabbit, ox, sheep, and pig, epinephrin constricts the coronary vessels in man and the monkey.

The coronary arteries of the last two species are presumably supplied with constrictor nerves of true sympathetic (thoracicolumbar) origin.

FIG 1.

FIG 2.

FIG.3.

FIG.4.

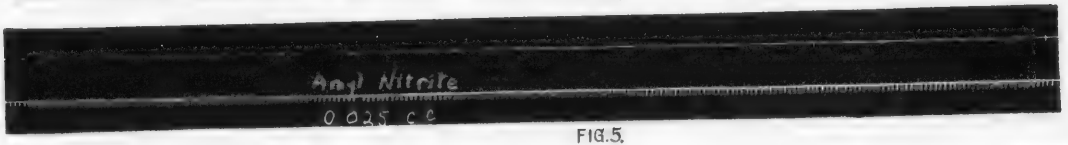

FIG.5.

(Barbour and Prince: Influence of Epinephrin upon Monkey.)

EXPLANATION OF PLATE 22.

Records of coronary perfusion. Upper line = perfusion units; lower line = time in five second intervals.

FIG. 1. Rabbit 1, 0.25 mg. of epinephrin.

FIG. 2. Monkey 1, 0.25 mg. of epinephrin. The numbers indicate the heart rate per ten seconds.

FIG. 3. Monkey 2, 2 mg. of epinephrin.

FIG. 4. Monkey 3, 0.1 mg. of epinephrin.

FIG. 5. Monkey 3, 0.025 c.c. of amyl nitrite.

A TEST FOR ANTITHROMBIN IN THE BLOOD.*

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The part that antithrombin plays in the human blood in inhibiting coagulation is a question concerning which there is a decided difference of opinion. Although it is admitted generally by physiologists that antithrombin is present in the circulating blood, there is a marked divergence as to the significance and importance which should be accorded it. On the one hand, we note that Morawitz, whose theory of coagulation has gained wide acceptance, assigns to antithrombin no function in the theory of coagulation which he has elaborated. This omission seems to be a weak link in the chain which he has constructed, as any theory is necessarily incomplete which leaves out of consideration a substance which is regularly present in the circulating blood. On the other hand, Howell considers antithrombin to be a very important constituent, ascribing to it the part of maintaining the fluidity of the blood, in that coagulation ensues only when the antithrombin is rendered inert by the neutralizing effect of the zymoplastic substance in the shed blood or in the tissues. As stated, however, there is no diversity of opinion regarding the normal occurrence of antithrombin, so that it would seem worth while to study this substance clinically from a quantitative point of view, in order to obtain fuller data from which to judge its importance. The term antithrombin is employed in a functional sense to designate any substance or substances in the plasma which tend to inhibit coagulation. It is realized that a terminology of this kind cannot be absolutely satisfactory, especially from a chemical point of view. Nevertheless, it appears to be justifiable; it is, for example, in accordance with the physiological studies in immunity, where all the substances,—complement, amboceptor, etc.,—exist only from a functional viewpoint.

There have been very few quantitative examinations of antithrom-

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bin in the human blood. This, probably, may be accounted for by the fact that it has been overshadowed by the striking importance of other substances in the blood,—the thrombin, fibrinogen, and other active principles, which may be grouped as the positive coagulative factors,—and in part by the fact that there has been no simple method for making such tests. The few estimations of this nature which have been carried out have been reported by Howell, who used a method which he has devised. Although this method seems to be satisfactory, it is by no means simple, and is hardly suited to wide clinical use. In the first place, it requires the preparation of a pure thrombin. This is extracted from pig fibrin by treating it with an 8 per cent. solution of sodium chloride, followed by repeated extractions of the coagulable proteins by means of chloroform. The test also requires the preparation of a solution of fibrinogen which must contain no prothrombin; that is, must not clot merely upon the addition of calcium. It requires considerable experience to prepare both of these substances satisfactorily; the fibrinogen solution, which is not the salted fibrinogen of Hammarsten, but a dialyzed plasma, is especially difficult to obtain and to maintain free from contamination of prothrombin.

In the course of testing the coagulability of oxalated plasma from various sources, an examination which included a test for antithrombin, it was found that for clinical use a simple method could be employed, which requires neither a preparation of thrombin nor of fibrinogen. The method is carried out as follows:

About nine cubic centimeters of blood are aspirated and put into one cubic centimeter of 1 per cent. sodium oxalate. The blood is centrifugalized and the plasma removed in the usual way. The plasma is then recalcified by adding 2, 3, 4, and 5 drops, respectively, of a 0.5 per cent. calcium chloride solution. In this way we ascertain the general coagulability of the plasma, which is the composite of a number of interacting factors,—prothrombin, fibrinogen, antithrombin, etc.—and we determine at the same time the optimal amount of calcium for this particular plasma.

If we heat some of this plasma to 60° C., the prothrombin, as is well known, is destroyed and the fibrinogen is coagulated. After filtering off the coagulum we have a plasma which contains anti-

thrombin and practically no prothrombin. The strength of this antithrombin may be ascertained, for clinical purposes, as follows:

First, we prepare human plasma from a normal case just as we prepared the oxalated plasma which is to be tested. Five drops of this plasma are put into five thoroughly cleansed vials. The first of these serves as a control; to the second three drops of normal antithrombin are added; to the third five drops of normal antithrombin; to the fourth three drops of the antithrombin that is to be tested; and to the fifth five drops of this antithrombin. All tubes are equalized in amount by the addition of normal salt solution, and the mixtures are allowed to remain in contact for fifteen minutes. The plasma is then recalcified by the addition of 0.5 per cent. calcium chloride, the number of drops which are added having been determined by the general coagulability test, which should always precede the antithrombin test. As a rule, four drops have been found to be the optimal amount.

TABLE I.

Antithrombin Test.

A.

Normal.			Hemophilia.		Interval.
Control.	3 drops antithrombin.	5 drops antithrombin.	3 drops antithrombin.	5 drops antithrombin.	
−	−	−	−	−	2 min.
+	+	+	+	+	4 min.
+++	+++	++	+++	++	6 min.
		+++		+++	8 min.

B.

Normal.			Purpura.		Interval.
Control.	3 drops antithrombin.	5 drops antithrombin.	3 drops antithrombin.	5 drops antithrombin.	
−	−	−	−	−	4 min.
+	+	+	+	+	6 min.
+	+	+	+	+	8 min.
+++	++	++	+	+	10 min.
	+++	++	++	++	12 min.
		++	+++	++	14 min.
		+++		+++	16 min.

Table I *A* illustrates a test of this kind in an atypical case of hemophilia where there was a deficiency of calcium in the blood. We note that antithrombin was present in the plasma of the patient to no

greater degree than in the normal plasma; in both instances coagulation was rapid and but slightly delayed by the addition of three and of five drops of antithrombin. Table I *B* illustrates a similar test in a case of purpura. Here we likewise find no increase in antithrombin.

Antithrombin is judged to be in excess where a marked delay in coagulation is brought about in the tubes to which it has been added, as compared with the coagulation in the control tube. As in the case of the coagulation time, no arbitrary norm can be set up; the results should be well defined to warrant the conclusion that there is an excess of antithrombin.

The essential difference between this antithrombin test and that of Howell is that plasma is used as a basis instead of a fibrinogen solution. The validity of employing plasma in this way may be determined by preparing solutions of hirudin of varying strengths and titrating them upon plasma; in other words, by substituting hirudin for human antithrombin. Tests of this nature were carried out, first upon horse plasma, and later upon normal human plasma. In both cases the plasma was clear and had a coagulation time of not over ten minutes. Table II *A* illustrates an experiment of this kind. We may note that dilutions of 1 to 20,000, 30,000, and 40,000 of hirudin were employed, and that in each instance 1, 3, and 5 drops of one of these antithrombin solutions were added to the human plasma. It will be seen that in each of the three tests the sequence of coagulation is in direct ratio to the number of drops of antithrombin added, and that in general the more dilute the hirudin, the less its inhibiting effect. A test (table II *B*) of normal human antithrombin upon its own plasma has been added to this table to enable a comparison between the strengths of the hirudin used and of human antithrombin. It will be seen that, according to the test, human antithrombin is about equal to a 1 to 40,000 solution of hirudin.

If we turn again to table I, we notice that in addition to the tests of plasma in the case of purpura and of hemophilia, it includes titrations of antithrombin upon autogenous plasma. In these instances the plasma was normal. The same autogenous test may be carried out in pathological cases; *e. g.*, in hemophilia, as shown in table III, which reproduces an equilibrium test of this kind with the

same plasma which is reported in table I. This test evidently can not be considered an antithrombin test, as we employ plasma from a pathological case in order to ascertain the degree of antithrombin. It is termed an equilibrium test because it gives us information as to the balance which obtains in the plasma between all positive and negative factors concerned in coagulation. If the balance is in a state of delicate adjustment, the addition of a small amount of antithrombin will suffice greatly to delay coagulation, whereas if there is an excess of prothrombin and allied substances, this addition will bring about but a slight increase in the coagulation time. We see this when we compare the two tables. In table I, where two equilibrium tests of normal plasma are shown, the addition of three drops of antithrombin resulted in either slight or no delay, and five drops brought about at most a retardation of from ten to sixteen minutes. These tests must not be considered exceptions to the rule, although the delay when three drops of antithrombin are added is generally more marked, and when five drops are added, the period of complete coagulation is postponed to fifteen or twenty minutes. On the other hand, with the plasma of the hemophiliac, referred to in table I, the addition of the same amounts of antithrombin was sufficient to retard coagulation markedly (table III). Comparative tests such as these demonstrate that the mere coagulation time does not furnish complete information as regards the power for clotting which exists in plasma. There are latent potential coagulative factors which come to light only when inhibiting substances are added to the plasma. For example, a normal plasma (table II *A*) coagulated in ten minutes; the plasma of the hemophiliac (table III) in twelve minutes, that is, almost in the same length of time; nevertheless, upon the addition of five drops of its own antithrombin, the former coagulated in sixteen minutes, whereas the latter took forty-three minutes to clot. This test is not recommended for clinical use, because the testing of antithrombin upon abnormal plasma is erroneous. However, it is highly significant from one point of view: it shows that the circulating blood is not delicately balanced in regard to its coagulability. When we reflect that in adding three and five drops of antithrombin to five drops of plasma we are more than doubling its normal content of antithrombin, and that never-

theless coagulation generally ensues with but slight retardation, we must conclude that there exists a considerable factor of safety in the mechanism of the coagulation of the circulating blood. If such were not the case, serious hemorrhage, as the result of a slight temporary excess of antithrombin, would be a constant danger.

TABLE II.

A. Hirudin.

Dilution 1 :20,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
−	−	−	5 min.
+	+	−	8 min.
++	+	+	10 min.
++	++	+	11 min.
++	++	+	12 min.
+++	++	++	14 min.
	++	++	16 min.
	+++	++	18 min.
		++	20 min.
		+++	22 min.

Dilution 1 : 30,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
−	−	−	5 min.
+	+	+	8 min.
++	++	+	10 min.
+++	++	++	11 min.
	++	++	12 min.
	+++	++	14 min.
		+++	16 min.

Dilution 1 : 40,000.

1 drop hirudin.	3 drops hirudin.	5 drops hirudin.	Interval.
−	−	−	5 min.
+	+	+	8 min.
+++	++	++	10 min.
	++	++	11 min.
	++	++	12 min.
	+++	++	14 min.
		+++	16 min.

B. Antithrombin Test.

Control.	1 drop antithrombin.	3 drops antithrombin.	5 drops antithrombin.	nterval.
+	+	+	−	5 min.
++	++	++	+	8 min.
+++	+++	+++	++	10 min.
			++	11 min.
			++	12 min.
			+++	14 min.

TABLE III.

Equilibrium Test.[1]

Control.	3 drops antithrombin.	5 drops antithrombin.	Interval.
−	−	−	6 min.
+	+	+	10 min.
+++	+	+	12 min.
	+++	+	15 min.
		++	35 min.
		+++	43 min.

SUMMARY.

A test is described for the estimation of antithrombin in the blood. The chief advantage of the test is that it is simple, and does not require the preparation of fibrinogen and of thrombin, which are difficult to prepare and to maintain in a pure state. The principle consists in titrating the antithrombin against normal human plasma; in this way we obtain an estimation of its power to delay coagulation. As the result of examinations carried out by this method, it would seem that there is a wide factor of safety as regards the amount of antithrombin in the human blood, and that this inhibiting substance may be increased to a considerable degree without markedly delaying or endangering clotting.

[1] The same case as in table I *A*.

CONCERNING CHANGES IN THE BIOLOGICAL PROPERTIES OF TRYPANOSOMA LEWISI PRODUCED BY EXPERIMENTAL MEANS, WITH ESPECIAL REFERENCE TO VIRULENCE.*

By WADE H. BROWN, M.D.

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During the past few years a great deal of interest has been manifested in biological variations, or mutations, in trypanosomes induced by experimental procedures. That even such simple manipulations as are practiced in maintaining a stock strain of trypanosomes might lead to an appreciable degree of biological alteration in some trypanosomes was rather strongly suggested to me by an instance of exalted virulence in a strain of *Trypanosoma lewisi,* an account of which was published about a year ago.[1]

While others have observed similar examples of virulence in *Trypanosoma lewisi,* notably Jürgens,[2] no satisfactory explanation has been offered for such occurrences. By experimental procedure, however, very marked alterations in the biological properties of this organism have been produced. As early as 1909 Wendelstadt and Fellmer[3] succeeded in producing morphological alterations and an increase in the virulence of *Trypanosoma lewisi* by a series of passages through cold-blooded animals. More recently, the experiments with a reinforced virus, initiated by Roudsky,[4] have demonstrated clearly the possibility of developing a strain of *Trypanosoma lewisi* of a highly virulent character, capable even of serial infection in mice.

Especial importance may be attached to the use of *Trypanosoma lewisi* in such studies on variation, as the relations obtaining between

* Received for publication, February 10, 1915.

[1] Brown, W. H., *Jour. Exper. Med.,* 1914, xix, 406.

[2] Jürgens, *Arch. f. Hyg.,* 1902, xlii, 265.

[3] Wendelstadt, H., and Fellmer, T., *Ztschr. f. Immunitätsforsch., Orig.,* 1909, iii, 422; 1910, v, 337.

[4] Roudsky, D., *Compt. rend. Soc. de biol.,* 1910, lxviii, 421, 458.

host and parasite in the instance of this organism are different from those existing with most laboratory strains of trypanosomes, in that *Trypanosoma lewisi* is one of a few constantly carried in its natural host. While seeking an explanation of our specific problem of virulence, therefore, the attempt was made to approach the problem from a fundamental biological viewpoint with the belief that such information as could be obtained might prove of broader application.

SOURCES AND NATURE OF MATERIALS.

In the particular instance of variation that occurred in our stock strain of *Trypanosoma lewisi,* the indications were that either the mode of passage, the character of the rats, or both factors had been instrumental in inducing the change. Accordingly, a simple series of experiments was begun to test the influence of the rate of passage, passage during different periods of the infection, the dose of trypanosomes used, and the character of the rats, as measured by weight, upon succeeding generations of trypanosomes. Two strains of *Trypanosoma lewisi* were used in the tests. One of these, designated as strain I, was the so called pathogenic strain, and the other, strain V, had just been obtained from a natural infection in a young rat. This rat was one of seven infected rats out of a lot of thirty-six obtained from a dealer. The infections in these rats had progressed without symptoms, indicating that the organism was of a relatively harmless type. Very distinct differences between these two strains of *Trypanosoma lewisi* persisted through four parallel series of experiments on white rats. The first series of rats, tables I and II, comprised the stock transfers. These rats were mostly large. They were all inoculated intraperitoneally with two drops of tail blood in 0.5 of a cubic centimeter of salt solution. The rate of passage was necessarily irregular but relatively slow, and in some instances there were several groups of rats inoculated from a common source infection at different periods of the infection, as in generations VI, VII, VIII, and X (table I).

In the second series (tables III and IV) large rats were also used. The inoculations were carried out as in the first series. The transfers were made, however, as near the height of multiplication as pos-

sible, except in the last five generations of strain V; the rate of passage was relatively rapid and uniform.

The third series of experiments, given in tables V and VI, differed from the second only in the use of small rats. The rate of passage in the last seven generations of strain V was intentionally varied here as in series 2.

In the fourth group small rats were used as far as possible (tables VII and VIII), and were inoculated in pairs. At the height of multiplication one animal was killed and its defibrinated blood, up to 0.5 of a cubic centimeter, was injected intraperitoneally into each of the next two rats. Thus the rats in the records do not represent a series of direct transfers but are, as it were, only the controls of the series.

In all these animals accurate records were kept of the course of infections. The blood was examined daily and weights were recorded at least once a week for thirty days. Observations were then discontinued in all except the stock series (series 1) where observations were continued until the termination of the infection.

CHANGES PRODUCED IN THE INFECTION CYCLE.

Incubation Period.—Other conditions being kept relatively constant, the rate at which *Trypanosoma lewisi* was passed from rat to rat was found to exercise a distinct influence upon the various phases of the infection cycle in succeeding generations of the organism. The earliest evidence of such an effect was seen in the incubation period. An examination of table I shows that of thirty-five rats in only three was the incubation period less than two days, and these rats had been inoculated from infections of seven and ten days' duration. There were six other rats in the series inoculated at relatively the same periods that showed longer periods of incubation. Again, with strain V, table II, we find three rats with incubation periods of one day. Here, however, two of these followed inoculation from an infection of twenty-nine days' duration. Again, there were three rats inoculated from infections of only ten days' duration, or less, with incubation periods of two days. That the tendency of rapid passage was undoubtedly to shorten the incubation period in suc-

TABLE I.

Slow Passage of Small Doses of Virus in Large Rats.

Trypanosoma lewisi, Strain I, Series 1.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	85	5th	5	++	Marked	11	Death	
II	2	175	10th	4	++	Marked	11	Death	
	* 3	132	10th	4	++	Marked	15	Death	
III	4	116	12th	5	+++	Marked	8	Death	
	* 5	150	12th	4	++	Marked	12	Death	
IV	* 6	146	8th	3	+++	Marked	—	Killed	Used to test virulence of strain. Killed after 12 dys.
	7	176	8th	—	—	—	—	—	Rats 7 and 8 immune.
	8	165	8th	—	—	—	—	—	
V	9	70	10th	2	+++	Marked	37	Killed	For bacteriological and pathological study.
	*10	72	10th	2	++	Moderate	107	Recovery	Late symptoms marked.
VI	11	79	13th	2	++	Moderate	—	Killed	To test virulence of strain.
	12	162	13th	4	+	Slight	22	Recovery	Experiments actually began with this generation.
	13	85	41st	5	+++	Marked	18	Death	
	14	79	61st	5	++	Moderate	14	Recovery	
	*15	127	61st	6	++	Slight	58	Recovery	
VII	*16	135	29th	5	+++	Marked	12	Death	
	17	93	46th	—	—	—	—	—	Immune.
	*18	127	46th	5	++++	Marked	49	Recovery	Condition fair.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
VIII	19	114	10th	1	+++	Marked	13	Recovery	Rats 19 and 20 inoculated from 16; others from 18. Rat 19, condition poor.
	20	63	10th	1	+++	Marked	23	Death	
	21	141	12th	2	++	Moderate	44	Recovery	Condition good.
	22	141	12th	2	++	Moderate	76	Recovery	Condition good.
	23	143	21st	3	++	Moderate	34	Recovery	Condition fair.
	24	92	21st	3	+++	Marked	22	Death	
	*25	145	32d	3	+++	Marked	35	Death	
IX	*26	150	7th	1	+++	Slight	61	Recovery	Condition good.
	27	140	18th	—	—	—	—	—	Immune.
X	28	146	28th	2	++	Slight	10	Recovery	Condition good.
	29	152	28th	2	+	Slight	10	Recovery	Condition good.
	*30	130	37th	6	+++	Marked	37	Death	
	*31	126	37th	5	+++	Moderate	130	Death	Late symptoms marked.
	32	110	41st	—	—	—	—	—	Immune.
	33	118	41st	5	+++	Moderate	32	Death	Late symptoms marked.
XI	34	123	27th	4	+++	Marked	21	Death	Rat 34 inoculated from 30.
	35	119	43d	5	+++	Marked	108	Death	Rat 35 inoculated from 31.

In all the tables where several rats are shown in one generation of transfers, the rats in the direct series are marked with an asterisk (*).

The figures in the column marked "day of passage" indicate the time elapsing between successive inoculations and refer particularly to the duration of infection in the rat from which a given rat was inoculated. All times estimated are from the time of inoculation.

Relative numbers of trypanosomes in the blood are indicated thus: Very few, +; few, ++; many, +++; great many, ++++.

cessive generations of trypanosomes may be seen by comparing this phase of the infection cycle of the rats in tables I and II with those in the tables following. Still, it must be recognized that the incubation period in any specific instance may be influenced to a great extent by such factors as the age of the infecting organism and the number of the organisms injected.

In this connection a distinction must be drawn between true and false incubation,—a fact that seems to have escaped attention in the literature. Where large numbers of trypanosomes are used to infect an animal, it is not uncommon to find organisms in the blood within a few hours. In the case of the adult forms of *Trypanosoma lewisi* it is easy to show that such an invasion of the blood is not a true incubation, as the type of organism is the same as that injected and the numbers of the organisms subsequently diminish or may entirely disappear from the blood for a day or more before the true invasion of young forms occurs. Where young or multiplying trypanosomes are used, the distinction between true and false incubation becomes arbitrary or fails.

Periods of Multiplication.—As with the period of incubation, rapid passage of *Trypanosoma lewisi* tends to advance the time at which multiplication begins in the blood of the rat. In the tabulated records of the rats in series 2, 3, and 4, the figure in the column indicating the "day of passage," in most instances, also indicates the day on which the height of multiplication was reached in the preceding rat. In many of these rats, multiplication was well advanced by the end of the second day after injection and completed by the end of the fourth day. This is in sharp contrast to the usual phase of multiplication, both as to time and duration of the cycle, as well as to the period elapsing between incubation and multiplication. As in the case of incubation, only a few passages were necessary to develop the tendency to shorten the gap between incubation and multiplication, to accentuate the rate of multiplication, and to favor an early termination of the cycle.

Duration of Infection.—Infections of *Trypanosoma lewisi* tend to be chronic, to last for thirty days or more, but exceptions to this rule are not at all uncommon; the usual termination is in recovery. In series 1 of strain V, after the first generation, only one rat out of

TABLE II.

Slow Passage of Small Doses of Virus in Large Rats.

Trypanosoma lewisi, Strain V, Series 1.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	* 1	117	?	4	+++	Slight	20	Recovery	Condition good.
	2	92	?	5	+++	Slight	22	Recovery	Condition good.
	3	83	?	5	+++	Slight	—	Killed	Killed 8th dy. to test virulence or strain.
II	4	90	4th	2	++++	Slight	86	Recovery	
III	5	90	23d	—	—	—	—	—	Immune.
	* 6	110	39th	3	++++	Moderate	47	Recovery	Condition good.
IV	7	93	34th	4	++++	Moderate	48	Recovery	Condition good.
V	8	153	29th	1	++	Moderate	59	Death	Rats 8 and 9 were infested with lice(?).
	9	145	29th	1	++	Moderate	80	Death	
	*10	167	38th	3	+++	Marked	34	Recovery	Condition poor.
VI	*11	147	15th	2	++++	Marked	14	Death	
	12	165	18th	—	—	—	—	—	Immune.
VII	*13	130	10th	2	+++	Moderate	35	Recovery	Condition fair.
	14	142	10th	2	+++	Moderate	35	Recovery	Condition fair.
VIII	15	154	21st	2	++++	Moderate	27	Recovery	Condition very poor.
IX	16	123	18th	2	++++	Moderate	31	Death	
	17	114	18th	—	—	—	—	—	Immune.
X	18	136	10th	1	+++	Moderate	33	Recovery	Condition poor.
	19	127	29th	2	++++	Marked	45	Recovery	Condition poor.

TABLE III.

Rapid Passage of Small Doses of Virus in Large Rats.

Trypanosoma lewisi, Strain I, Series 2.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	111	7th	2	+	Moderate	10	Recovery	Virus from rat 11, series 1. Condition fair.
II	2	117	6th	4	+	Marked	10	Recovery	Condition poor.
III	3	135	7th	2	+++	Marked	30+	Survival	Condition good.
IV	4	211	4th	2	+++	Moderate	30+	Survival	Condition fair.
V	*5	94	4th	1	++	Slight	15	Recovery	Condition fair.
	6	95	4th	1	++	Slight	22	Recovery	Condition fair.
VI	7	102	5th	2	+	Slight	11	Recovery	Condition fair.
VII	8	200	4th	1	++	Slight	30+	Survival	Condition good.
VIII	9	122	3d	2	+++	Slight	30+	Survival	Condition good.
IX	10	150	4th	1	+	Marked	6	Recovery	Condition poor.
X	11	114	3d	3	+	Marked	13	Recovery	Condition poor.
XI	12	216	3d	5	++	Marked	12	Recovery	Inoculated from rat 13, series 3. Loss of weight, 46 gm. in 12 dys.

sixteen recovered within the thirty day period of observation (table II). In series 2, 3, and 4, however, where rapid passage was practiced, the last four rats, seven of the last nine, and five of the last six, respectively, recovered within the thirty day period.

In strain I the influence of passage upon the duration of the infection was not so clearly shown and there was only one series of rats, series 2, in which any consistent or definite course of alteration was manifest when compared with series 1. Other factors, which will be considered later, undoubtedly served to mask much of the effect of passage in this strain.

The results obtained with strain V, however, show very clearly what changes may be produced in the several phases of the infection cycle of an ordinary strain of *Trypanosoma lewisi* by the use of only the simplest experimental procedures. Mere regulation of the rate of passage of the virus from one rat to another seems sufficient so to alter the biological properties of *Trypanosoma lewisi* as to change an infection that is usually chronic into one that may be regarded as acute, or *vice versa*. These changes occurred, in my experiments, irrespective of the character of the rats used or of the dose of the infecting organisms.

CHANGES IN VIRULENCE.

The basis for estimating the virulence of *Trypanosoma lewisi* in these experiments was the incidence, especially serial incidence, and degree of such symptoms of intoxication as stupor, weakness, loss of weight, and anemia, together with the mortality definitely attributable to infection with *Trypanosoma lewisi;* very little account was taken of isolated instances of severe intoxication or even death.

In series 1 of strain I, the records show that infections with marked symptoms and even death occurred in all except one of the eleven generations of transfers. The virulence of the strain was at its height in the first three generations, though strongly evident again in the last two generations.

In the second series of large rats where the virus was passed rapidly, virulence was much less evident than in series 1. There were, however, well marked symptomatic disturbances in the first

three rats of the series. Even symptoms were then absent until the last two generations when they again became marked. The young rats of the third series showed a larger proportion of severe infections and a few deaths. Crossed infection at the termination of series 2 and 3 showed, however, that there was no appreciable difference in the character of the trypanosomes in the two series at that time (compare rats 11 and 12, series 2, and 13 and 14, series 3). The added influence of large doses of trypanosomes, as in series 4, produced no further alteration in the character of the infection except to insure a consistently shortened incubation period and a greater number of trypanosomes in the peripheral blood. No pronounced virulence was developed by strain V within the limits of these experiments, although the rats of series 1 and 3 showed a gradually increasing number of infections with distinct evidence of intoxication. This was especially evident in the last half of series 3, where the rate of passage was changed with a view to increasing the virulence of the organism. To a less degree, the other series showed a similar increase. The fluctuation in the character of the infections throughout was such as to suggest a cyclic series of changes,—a condition evident also with strain I.

A final factor that appeared to exercise a considerable influence upon the nature of the infection produced by *Trypanosoma lewisi* was the time at which the transfer of the trypanosomes was made. From table I it will be seen that in generation VI three sets of rats were inoculated. Two rats, inoculated from an infection of thirteen days' duration with active agglomeration in progress, developed mild infections. Another rat, inoculated from the same source on the forty-first day of infection, developed a severe infection which terminated fatally, while two other rats inoculated from the same rat on the sixty-first day of infection developed relatively mild infections. A similar condition was observed in generation VIII. Rats 19 and 20, inoculated from rat 16, both showed severe infections. One of these rats recovered after an acute infection and the other succumbed. Rats 21 and 22, inoculated from rat 18 on the twelfth day of infection, developed comparatively mild infections, while the infections in two of three other rats, inoculated from the

TABLE IV.

Rapid Passage of Small Doses of Virus in Large Rats.

Trypanosoma lewisi, Strain V, Series 2.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	94	4th	2	++++	Slight	30+	Survival	Virus from rat 3, series 1. Condition good.
II	2	92	4th	1	++++	Slight	30+	Survival	Condition good.
III	3	90	5th	2	++++	Slight	30+	Survival	Condition good.
IV	4	93	4th	1	++++	Moderate	30+	Survival	Condition fair.
V	5	140	3d	1	+++	Slight	30+	Survival	Condition good.
VI	6	115	4th	1	+++	Slight	30+	Survival	Condition good.
VII	7	174	3d	1	+++	Slight	30+	Survival	Condition good.
VIII	8	215	3d	2	+++	Moderate	15	Death	
IX	9	210	4th	1	+++	Marked	—	Killed	Killed after 7 dys. to test virulence of strain.
X	10	217	4th	1	+++	Moderate	28	Recovery	Condition fair.
XI	11	145	3d	1	++++	Slight	30+	Survival	Condition fair.
XII	12	137	4th	1	+++	Marked	18	Death	
XIII	13	132	5th	1	++	Moderate	30+	Survival	Condition fair.
XIV	14	140	6th	2	+++	Moderate	18	Recovery	Condition fair.
XV	15	122	8th	2	++++	Moderate	14	Recovery	Condition poor.
XVI	16	143	8th	2	+++	Moderate	14	Recovery	Condition fair.
XVII	17	110	11th	4	++	Moderate	28	Recovery	Condition poor.

same source on the twenty-first and thirty-second days of infection, terminated fatally. Again, in generation X similar results were obtained.

Similar experiments with strain V were inadequate to warrant independent interpretation, but, as far as they went, they were in harmony with those obtained with strain I.

CHANGES IN MORPHOLOGY.

This phase of the subject of biological variation of *Trypanosoma lewisi* has been discussed in detail in another paper.[5] It seems necessary, therefore, only to point out that the variations of morphology observed occurred mainly in infections of considerable severity, indicating a measure of interrelation between virulence and morphological variation.

Before entering upon a discussion of the experiments already described, mention should be made of still another group of experiments, the details of which must be omitted on account of some uncertainty as to their end results. This series of experiments comprised two groups of rats, large and small, with each of the two strains of *Trypanosoma lewisi.*

In these rats the infection was allowed to progress until recovery seemed imminent, when the rat was killed and 0.5 of a cubic centimeter of its defibrinated blood injected into the next rat. With the large rats of both strains and with the small rats of strain I, the trypanosomes were propagated for only a few generations when infection failed, presumably due to the use of immune rats. Two of the series had been interrupted in this manner before it occurred to us to test the immunity of these rats to the stock strain. When infection failed to develop in the third series, after two weeks, this rat was inoculated with the stock strain of *Trypanosoma lewisi* and promptly developed an infection. The possibility of an attenuation by the slow passage of large doses of trypanosomes was neither excluded nor proven, but the most plausible explanation of this result seems to be the transference of sufficient immune bodies with the trypanosomes to protect the new host against infection. This point raises a question of practical importance as to the propriety of a procedure so often employed in attempting to recover trypanosomes

[5] Brown, W. H., *Jour. Exper. Med.*, 1914, xix, 562.

TABLE V.

Rapid Passage of Small Doses of Virus in Small Rats.

Trypanosoma lewisi, Strain I, Series 3.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	93	7th	2	+	Moderate	10	Recovery	Virus from rat 11, series 1. Condition poor.
II	2	91	6th	5	+	Marked	10	Recovery	Condition poor.
III	3	60	7th	3	+++	Marked	30+	Survival	Condition poor.
IV	4	73	4th	2	+++	Marked	—	Killed	Killed after 5 dys. to start series 4.
V	5	70	4th	1	++++	Moderate	30+	Survival	Condition fair.
	*6	68	4th	1	++++	Moderate	30+	Survival	Condition fair.
VI	7	46	5th	1	+++	Marked	26	Death	
VII	8	60	3d	1	++	Moderate	30+	Survival	Condition fair.
	9	60	4th	1	++	Moderate	30+	Survival	Condition fair.
VIII	10	79	3d	2	+	Slight	30+	Survival	Condition good.
IX	11	46	4th	2	++	Marked	20	Death	
X	12	39	3d	1	+++	Slight	22	Recovery	Condition good.
XI	13	51	3d	1	++	Marked	7	Death	
XII	14	63	5th	2	+++	Marked	6	Death	Inoculated from rat 11, series 2.

from an animal suspected of being infected; namely, the transference of large amounts of blood containing but few trypanosomes and an unknown amount of immune bodies. Unfortunately, we have been unable to investigate this subject further.

DISCUSSION.

The experiments have necessarily covered a wide field of investigation in order to supply *indications* of the influence of a number of simple factors upon the biological properties of *Trypanosoma lewisi,* and must be so viewed. The conclusions that may be reached on some points are quite clear, while on others there is still some uncertainty.

It is perfectly obvious that, within certain limits, by regulating the time and rate at which the trypanosomes are passed from rat to rat, we may so alter the character of *Trypanosoma lewisi* as to change the course of the infection cycle completely. The development of a strain producing short or acute infections can be accomplished with greater certainty than the intentional reversion of such a strain to a consistently chronic type, or the maintenance of a chronic strain as such; the single factor of rapid passage seems sufficient to accomplish the transformation of a chronic into an acute strain, while in the development or maintenance of a chronic strain the essential conditions are more difficult of control. The occasional short infection in an exceptionally resistant rat, or the occasional severe infection in a susceptible rat, may compel an earlier transfer than would be desirable. Still more difficult to encompass are the cyclic changes in the character of the trypanosomes produced by immunological reactions in the rat's blood during the chronic phase of infection. In a broad sense, the biological status of the trypanosomes at any particular time during an infection may be regarded as only a resultant of these immunological reactions. Since the phenomenon of agglomeration constitutes our only guide to these reactions, the choice of a time when the biological status of the trypanosomes favors our purpose becomes a matter of extreme difficulty.

When we come to consider the question of virulence, this constantly changing status of *Trypanosoma lewisi* in the rat's blood

TABLE VI.

Rapid Passage of Small Doses of Virus in Small Rats.

Trypanosoma lewisi, Strain V, Series 3.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	55	4th	2	++	Slight	——	Killed	Virus from rat 3, series 1. To start series 4.
II	2	78	4th	1	+	Slight	30+	Survival	Condition good.
III	3	51	5th	2	+++	Marked	10	Death	
IV	4	62	4th	1	++++	Marked	30+	Survival	Condition poor.
V	5	62	3d	1	++++	Marked	30+	Survival	Condition fair.
VI	6	41	4th	1	++++	Moderate	30+	Survival	Condition fair.
VII	7	41	3d	1	++++	Marked	28	Death	
VIII	8	38	7th	1	++++	Marked	27	Death	
IX	9	43	4th	1	++++	Marked	30+	Survival	Condition very poor.
X	10	48	3d	1	++++	Moderate	25	Recovery	Condition good.
XI	11	39	4th	1	+++	Slight	24	Recovery	Condition good.
XII	12	49	5th	1	+++	Moderate	24	Recovery	Condition good.
XIII	13	42	6th	1	++++	Marked	18	Recovery	Condition fair.
XIV	14	38	8th	1	++++	Marked	20	Recovery	Condition fair.
XV	15	54	12th	1	+++	Moderate	21	Recovery	Condition fair.
XVI	16	74	8th	2	++++	Moderate	21	Recovery	Condition good.
XVII	17	63	3d	1	+++	Marked	26	Death	
XVIII	18	65	18th	2	++++	Marked	7	Death	

assumes even greater importance. Apparently the conditions to be met in maintaining or building up a virulent strain of *Trypanosoma lewisi* are indicated by the conditions obtaining in severe infections, of which I have observed three main types.

In one small group of such infections large numbers of trypanosomes persist in the blood with but scant evidence of their destruction. These infections are chronic and differ from the usual infection only in degree.

In the second and largest group the number of trypanosomes is never great, and degeneration and phagocytosis of trypanosomes are comparatively prominent. These infections may terminate very early,—usually in recovery,—or when multiplication persists beyond the accustomed limits the infection is more prolonged and not infrequently terminates fatally.

In the third and most important group multiplication is extremely active and irregular in character; the blood swarms with trypanosomes, and degeneration and disintegration of trypanosomes are marked; there is a marked leucocytosis, and the blood contains a large number of active phagocytes. Death of the rat usually occurs while multiplication is still active; recovery is exceptional.

If the last group be taken as the highest development of the virulent type, three factors in the infection become significant: vigorous reproduction, limited vitality of the trypanosomes, and a strong defensive (?) response on the part of the host. The association of vigorous reproduction with weakened resistance to destruction may appear somewhat paradoxical. There appears to be some distinction, however, between the mechanism limiting multiplication and that causing the destruction of trypanosomes in the rat's blood. In the usual infection of *Trypanosoma lewisi* we have evidence of what I must regard as two distinct classes of immunological reaction, one of which is concerned in checking the multiplication of trypanosomes, as ordinarily understood, and the other in their destruction. That these phenomena are in reality separable is shown by the fact that in some infections multiplication in the peripheral blood may be completely checked early in the infection with no appreciable decrease in the numbers of trypanosomes or other evidence of their destruction for weeks or even months. On the other hand, in certain infections, as indicated above, active multiplication may

TABLE VII.

Rapid Passage of Large Doses of Virus in Small Rats.

Trypanosoma lewisi, Strain I, Series 4.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	74	5th	1	+++	Slight	30+	Survival	Virus from rat 4, series 3.
II	2	102	4th	1	+++	Slight	30+	Survival	
III	3	57	2d	1	++++	Marked	30+	Survival	Condition very poor.
IV	4	200	2d	1	++++	Marked	17	Death	
V	5	62	3d	1	+++	Slight	30+	Survival	Condition good.
VI	6	58	2d	1	+++	Slight	30+	Survival	Condition good.
VII	7	67	2d	1	++++	Moderate	25	Recovery	Condition fair.
VIII	8	63	3d	1	+++	Slight	30+	Survival	Condition good.
IX	9	48	2d	1	+++	Slight	30+	Survival	Condition good.
X	10	70	3d	1	+++	Moderate	30+	Survival	Condition poor.
XI	11	61	3d	1	+++	Marked	6	Death	
XII	12	53	3d	1	+++	Slight	25	Recovery	Condition fair.
XIII	13	48	3d	1	+++	Slight	30+	Survival	Condition fair.
XIV	14	64	4th	1	+++	Moderate	30+	Survival	Condition fair.
	15	56	4th	1	+++	Moderate	30+	Survival	Condition fair.

continue beyond its usual limits, while the number of trypanosomes in the peripheral blood continually diminishes with abundant evidence of vigorous destruction.

The essential elements of a spontaneously acquired virulence of *Trypanosoma lewisi* appear, therefore, to be closely related to immunological reactions and to be dependent upon an appreciable degree of reproductive fastness, strong antigenic properties, and weakened resistance to destruction. Such an hypothetical basis for a naturally acquired virulence or for a virulence developed in *Trypanosoma lewisi* as the result of a system of passage obviously constitutes a state of unstable balance of the biological properties of the organism. It is not inconceivable, however, that such a condition might be maintained or developed by a fortunate system of passage that takes due account of the existing biological status, or balance, of the organism to be dealt with. For example, strain V of my experimental series was a normally balanced strain and showed a consistent response to the experimental procedures employed. Strain I, on the contrary, was highly unstable so that under the same experimental conditions as strain V its fluctuations were frequent and sharp with but little persistent tendency to change in any given direction.

Moreover, reference should be made to the animal equation in experimental modification of *Trypanosoma lewisi.* As far as resistance to infection is concerned, small rats seemed to offer less resistance or were more susceptible to intoxication than large rats, as others have repeatedly observed. Theoretically, it should be possible to utilize this factor, since in its final aspects the problem is one of host and parasite, with the host supplying the mechanism through which the desired result may be accomplished; it is this feature of the reaction with which we are least able to reckon.

The extent of these experiments does not permit of deductions as to the permanency of any of the acquired properties of *Trypanosoma lewisi* that have been discussed. The tendency to revert to the natural or stable form of balance was quite evident in all instances as far as my experiments went, but much more extended series would be necessary before an opinion could be expressed as to the impress left upon *Trypanosoma lewisi* by such experimental procedures.

TABLE VIII.

Rapid Passage of Large Doses of Virus in Small Rats.

Trypanosoma lewisi, Strain V, Series 4.

Generation.	No.	Weight in gm.	Dy. of passage.	Incubation period in dys.	Trypanosomes in blood.	Symptoms of infection.	Duration of infection in dys.	Termination of infection.	Remarks.
I	1	73	5th	1	++++	Slight	30+	Survival	Series started from rat 1, series 3.
II	2	96	4th	1	++++	Slight	30+	Survival	Condition good.
III	3	51	2d	1	++++	Moderate	30+	Survival	Infection very light and condition poor at 30 dys.
IV	4	200	2d	1	+++	Marked	22	Death	
V	5	75	1st	1	++++	Slight	30+	Survival	Condition good.
VI	6	62	2d	1	++++	Moderate	30+	Survival	Condition fair.
VII	7	54	2d	1	++++	Moderate	30+	Survival	Condition poor.
VIII	8	92	2d	1	++++	Slight	30+	Survival	Condition good.
IX	9	72	2d	1	++++	Slight	30+	Survival	Condition good.
X	10	68	2d	1	++++	Slight	30+	Survival	Condition good.
XI	11	63	3d	1	+++	Slight	30	Recovery	Condition fair.
XII	12	56	3d	1	+++	Moderate	22	Recovery	Condition poor.
XIII	13	47	3d	1	+++	Moderate	28	Recovery	Late symptoms marked.
XIV	14	38	3d	1	++++	Marked	30+	Survival	Condition very poor.
XV	15	53	4th	1	+++	Moderate	29	Recovery	Condition good.
	16	61	4th	1	+++	Slight	18	Recovery	Condition good.

In conclusion, since the object of the investigation was to obtain indications of the nature and mode of action of the factors influencing the biological properties of *Trypanosoma lewisi,* the results must be interpreted in a broad sense, as much of the detail requires long and careful study. The experiments have served, however, to give a clearer insight into the relations existing between *Trypanosoma lewisi* and its host and afford a basis for formulating a conception of the essential elements of the virulence of this organism. The results also indicate that immunological reactions of two distinct types exercise a dominant influence as determinative factors in natural and experimental modifications of the species, and that by judicious control of these forces marked changes in the biological properties of *Trypanosoma lewisi* may be accomplished.

SUMMARY.

1. Different strains of *Trypanosoma lewisi* represent different states of biological balance, especially between the powers of propagation and resistance to destruction.

2. The biological status of a given strain of *Trypanosoma lewisi* is subject to cyclic variations as the result of immunological reactions in the blood of the host.

3. The factors limiting reproduction and causing destruction of *Trypanosoma lewisi* in the blood are appreciably independent of each other. It is possible, therefore, to influence these processes separately and even in opposite directions.

4. The virulence of *Trypanosoma lewisi,* manifested in its highest form, is dependent upon some degree of reproductive fastness, strong antigenic action, and susceptibility to destruction, varying degrees in the development of these properties producing corresponding variations in the degree of virulence.

5. By a properly regulated system of passage the properties of *Trypanosoma lewisi* that determine its infection cycle and its virulence may be eventually so altered as to change completely both the nature and course of the infection. Such a system of passage must be adapted to the particular strain of *Trypanosoma lewisi* used.

6. Immunological reactions exercise a dominant influence in determining the ultimate biological variations of *Trypanosoma lewisi.*

THE INFLUENCE OF MILK FEEDING ON MORTALITY AND GROWTH, AND ON THE CHARACTER OF THE INTESTINAL FLORA.*

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I. THE INFLUENCE OF MILK FEEDING ON MORTALITY AND GROWTH.[1]

The investigations which furnish the data presented in this paper extended over a period of about three years. The milk feeding experiments were the direct outcome of the researches that had been conducted for several years at the Storrs Agricultural Experiment Station on bacillary white diarrhea of chicks, and had as their chief object a study of the value of sour milk as a possible preventive and even curative agent in the elimination of bacillary white diarrhea. It was anticipated, in conformity with the views of Metchnikoff and his followers, that sour milk, in virtue of the acids that it contains, or as the immediate result of the acid-producing bacteria, might exert a beneficial influence in preventing or allaying the disease, if supplied soon enough.

The results of the first year were such as to leave little doubt as to the life-saving value of sour milk, in as far as bacillary white diarrhea was concerned. What appeared to be of far greater significance, however, was the marked influence of sour milk on the growth of the chicks and on the total death rate or mortality from all causes. In subsequent feeding experiments milk that was soured by *Bacillus bulgaricus* and sweet milk were employed, as well as ordinary sour

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[1] For references on the subjects of diet and growth, the reader is referred to the publications of Osborne and Mendel (Osborne, T. B., and Mendel, L. B., *Carnegie Institution of Washington Publications,* 1911, No. 156. Osborne, T. B., Mendel, L. B., and Ferry, E. L., *Ztschr. f. physiol. Chem.,* 1912, lxxx, 307; *Jour. Biol. Chem.,* 1912, xii, 81, 473; 1912–13, xiii, 233. Osborne, T. B., Mendel, L. B., Ferry, E. L., and Wakeman, A. J., *Jour. Biol. Chem.,* 1913, xv, 311; 1913–14, xvi, 423; 1914, xvii, 325, 401).

milk. Throughout the different series of experiments[2] the plan of investigation was essentially the same, and but for certain minor details was as follows:

The chicks, with few exceptions, were hatched in artificial incubators under as nearly uniform conditions as possible. When taken from the incubators the apparently sound chicks were divided uniformly into different lots, usually six. The number in each lot varied in the different experiments from fifteen to sixty. When sour milk was the only milk that was fed, as in the first series, the different lots or pens received the following treatment: Pen 1 was artificially infected with a bouillon culture of *Bacterium pullorum* when 24 to 36 hours old, and was fed sour milk; pen 2 was similarly infected, but received no milk; pen 3 was infected when 48 to 60 hours old and was fed sour milk; pen 4 was infected at the same age as pen 3, but was given no milk; pen 5 served as an uninfected control with sour milk, and pen 6 as an uninfected control without the milk.

In the numerous experiments which involved the use of two kinds of milk, as for example sweet and ordinary sour milk, the chicks were divided into equal lots, usually six. Pens 1, 2, and 3 were artificially infected with *Bacterium pullorum,* while pens 4, 5, and 6 were left uninfected. Pens 1 and 4 were fed the ordinary sour milk; pens 2 and 5 received the sweet or *bulgaricus* milk, according to the prearranged plan, and pens 3 and 6 went without milk. Thus double sets of controls were provided for.

Artificial infection with *Bacterium pullorum,* the organism which causes bacillary white diarrhea in chicks, was brought about by the use of a forty-eight hour bouillon culture which was administered with the aid of a medicine dropper. Three to six drops of the culture were placed in the beak in such a manner that the chick was compelled to swallow them. The milk was supplied in shallow galvanized pans having a capacity of about one pint. In order to prevent the chicks from wading in the pans coarse mesh wire was fastened over the tops. The pans were thoroughly cleaned at least

[2] Rettger, L. F., Kirkpatrick, W. F., and Stoneburn, F. H., *Bull. Storrs Agricultural Experiment Station,* 1912, No. 74. Rettger, L. F., Kirkpatrick, W. F., and Jones, R. E., *ibid.,* 1914, No. 77. Rettger, L. F., Kirkpatrick, W. F., and Card, L. E., *ibid.,* 1915, No. 80.

once a day, and scalded at definite intervals. In most of the experiments the milk was supplied twice a day.

Ordinary sour milk was usually obtained by adding a starter (sour milk) to fresh skimmed milk the day before it was to be used. The milk was kept at ordinary summer room temperature (25°–30° C.) either in pails or in milk bottles. *Bulgaricus* milk was prepared by sterilizing fresh skimmed milk, and after sufficient cooling, inoculating it with pure milk cultures of *Bacillus bulgaricus* which was procured in powder or tablet form from two reliable sources. The acidity was determined from day to day by titration. As a rule, the acidity was not permitted greatly to exceed 1.0 per cent. (in terms of lactic acid), since less of the milk was consumed when the acidity was high. The *bulgaricus* milk had the appearance of the product that is usually obtained with the use of *Bacillus bulgaricus*. Junket tablets were added in all but two instances to the sweet milk, in order to make it appear more attractive to the chicks. The conclusion was finally arrived at, however, that the junket tablets were unnecessary.

All the chicks were constantly supplied with an excess of feed. This consisted of the ordinary chick feed during the first week, after which a standard dry mash was fed. After the first year the feed going into each pen was weighed, and the amount left in the pans again weighed at the end of definite periods. The milk was also weighed, and complete records were kept of the milk and feed consumed per week.

The chicks were weighed at the beginning of the experiments, and once a week at as nearly the same hour as was possible. Daily mortality records were kept, and postmortem examinations were made of the chicks that died during the course of the experiments. Special emphasis was placed on the recovery of *Bacterium pullorum* from the internal organs and from the unabsorbed yolk.

In the entire investigation 5,118 chicks were employed. The majority of them were white Leghorns, though all the experiments of 1913 were conducted on white Plymouth Rocks. The small number of Rhode Island Red chicks that was used was of comparatively low vigor and was, therefore, less satisfactory than the others. The chicks were housed in small outdoor brooder houses which opened into large yards or runs, and in a large brooder house

from which long runs extended from each pen. About the same number of experiments was conducted under the two sets of housing conditions. The yards to which the chicks had access contained sufficient grass to supply them with the necessary amount of green food. The experiments were continued until the chicks were six to seven weeks old, when they were removed to permanent quarters.

RESULTS OF THE MILK FEEDING EXPERIMENTS.

Since no records of the amounts of feed and of sour milk consumed were kept in the investigations of the first year, the results are of far less importance than those of the two following years. Furthermore, the lack of complete uniformity in the plans of the different experiments makes the averaging of results somewhat difficult and unsatisfactory. It was most clearly shown throughout the work, however, that the feeding of sour milk exerted a beneficial influence on the growth and mortality of the chicks. The following tabulated results (table I) of experiment F will serve as a good illustration.

TABLE I.

	Infected when 36 hrs. old.		Infected when 60 hrs. old.		Uninfected controls.	
	Pen 1. Fed sour milk.	Pen 2. No milk.	Pen 3. Fed sour milk.	Pen 4. No milk.	Pen 5. Fed sour milk.	Pen 6. No milk.
Weight per 10 chicks.....	5.45 lbs.	3.07 lbs.	4.75 lbs.	3.22 lbs.	5.64 lbs.	3.14 lbs.
Mortality.....	3 or 8.1%	8 or 21.6%	2 or 5.4%	6 or 16.2%	0	4 or 10.8%

The total number of chicks used in the experiment was 222, or 37 in each pen.

In several instances the weight of the milk-fed chicks was, at the end of the experiment, at least double that of the chicks that received no milk, whether they were infected with *B. pullorum* or not. The sour milk chicks were stronger and more vigorous than the others, particularly in the uninfected pens. This was shown in different ways, as for example in the size and color of the combs, the strength of the feet and legs, and in the various activities of the chicks.

The sour milk feeding exerted a decided influence on mortality from bacillary white diarrhea as well as from all causes. In all of five complete experiments the mortality of the infected chicks was lower in the pens that were supplied with milk than in those which were not. In several instances the number of deaths in the lots with no milk was at least twice as great as in the corresponding pens that received milk, and in one case the ratio was approximately 3 : 1. Since the investigation of this first year involved the use of 1,044 chicks, some importance should be given to these results. In two experiments that were successfully carried through, and in which there were uninfected control lots, it happened that not a single death occurred in the sour milk pens that were not artificially infected with *B. pullorum,* while in the corresponding lots with no milk the mortality was approximately 11 per cent.

The results of the first year are in perfect harmony with those of subsequent investigations, in so far as the influence of milk feeding on growth and general

mortality is concerned. The apparent discrepancies that exist in the data of 1912 and 1913, bearing on the influence of milk feeding on the mortality from white diarrhea, may be fully explained, as will be seen later.

As none but ordinary sour milk was employed in the above experiments, no satisfactory explanation presented itself as to how such significant results of a sour milk diet could be brought about. It seemed to be of considerable importance, therefore, to determine, if possible, to what factor or factors milk owed these properties. It was for this purpose largely that the extensive investigations of the following two years were carried on. More complete data were sought, also, on the value of milk feeding as such.

In the second series of investigations seven different experiments were carried on and successfully completed. In three of them ordinary sour milk was the only milk that was supplied, while in the remaining four both the naturally soured and *bulgaricus* milk were used. Altogether 1,824 chicks were employed. In the first three experiments full records were made of the total gain in weight per ten chicks, the total feed consumed, the gain in weight per lb. of feed, and the mortality. In three of the four experiments in which a comparative study was made of ordinary sour and of *bulgaricus* milk the following data were acquired: total gain per ten chicks, total feed consumed, total milk consumed, total solid matter fed including the milk, per cent. acid in milk, gain per ten chicks for each lb. of solid food, and mortality. As the fourth experiment was conducted on hen-hatched and hen-reared chicks, no food records could be kept.

Time and space do not permit of a complete survey of the results here;[3] hence only brief summaries will be given.

TABLE II.

The Influence of Sour Milk Feeding on the Mortality of Infected and Uninfected Chicks.

Experiment.	No. of chicks in each pen.	Total mortality.			
		Infected chicks.		Uninfected chicks.	
		Fed sour milk.	No milk.	Fed sour milk.	No milk.
A	37	23	20	17	27
	37	23	20		
B	54	16	12	8	17
	54	7	13		
C	50	29	39	2	12
	50	18	16		
D	50	7	8	0	31
E	15	3	4	0	0
F	53	18	27	6	8
G	45	25	28	11	16
Total[4]		169, or 38%	187, or 42%	37, or 12.2%	111, or 36.5%

[3] For detailed statements of the results, including tables and curves, the reader is referred to Rettger, Kirkpatrick, and Jones, *loc. cit.*

[4] 445 in each of the two infected groups, and 304 in each of the two uninfected groups.

While the difference in the mortality of the two general infected groups (one supplied with sour milk and the other not) is but 4 per cent., the influence of sour milk feeding on mortality from all causes is encouraging (table II). Of the 304 uninfected chicks that were supplied with sour milk 37, or 12.2 per cent., died, as compared with a mortality of 36.5 per cent. in the corresponding group that received no milk. In other words, there were only one-third as many deaths in the former as in the latter. The mortality as a whole in the uninfected lots is considerably greater than it was during the previous year. This is undoubtedly due to a difference in the vitality of the chicks. In the investigations of 1912 strong white Leghorn stock was usually employed, while in the subsequent year white Plymouth Rock chicks which possessed no unusual vigor served as the subjects of investigation.

In the investigations of 1913 the chicks which were artificially infected with bouillon cultures of *B. pullorum* were subjected to the infecting process only once; that is, aside from the possibilities of natural infection between chicks, the chicks received but one treatment with the organism, and that was at practically the same time that the sour milk was supplied.[5]

TABLE III.

Combined Data Showing the Influence of Sour Milk Feeding on Growth.

	No. of chicks in experiments.	Gain per ten chicks.		
		Fed sour milk.	No milk.	Difference.
Infected lots.....	890	7.0 lbs.	4.3 lbs.	3.7 lbs., or 38.5%
Uninfected lots...	608	7.9 lbs.	4.6 lbs.	3.3 lbs., or 41.8%

The above summaries (table III) do not include any data on the feeding of *bulgaricus* milk. They are based on work that was in a large measure merely a continuation of the investigation of the previous year. In the following condensed statements the results of the feeding of milk that was soured by *B. bulgaricus* are included, and will therefore serve to show the relative merits of the two methods (table IV).

TABLE IV.

Gain Per Ten Chicks for Each Pound of Solid Matter, Including Milk Solids, Consumed.

	No. of chicks in experiments.	Gain per lb. of solid matter consumed.		
		Fed sour milk.	Fed *bulgaricus* milk.	No milk.
Infected lots.....	444	0.29 lb.	0.30 lb.	0.29 lb.
Uninfected lots...	444	0.32 lb.	0.30 lb.	0.26 lb.
Combined average	888	0.305 lb.	0.30 lb.	0.28 lb.

[5] The term sour milk is used throughout this paper to designate ordinary or naturally soured milk, in distinction from *bulgaricus* milk.

It is of interest to note that in the sour milk and the *bulgaricus* milk groups the gains per lb. of solid matter consumed are practically the same. In the lots that were not supplied with milk the average gain was noticeably less (approximately 8 per cent.). This difference, when taken by itself, is too small to be of much significance, but when considered along with other data should have some importance.

In order to make a comparative study of sour and of *bulgaricus* milk with special emphasis on acidity as an important factor, it was necessary to keep a full record of the acidity of both the sour and the *bulgaricus* product, and of the amounts fed. In table V the acidity is given in terms of lactic acid.

TABLE V.

Amounts of Acids in Milk Consumed.

Experiment.	Infected chicks.		Uninfected chicks.	
	Fed sour milk.	Fed *bulgaricus* milk.	Fed sour milk.	Fed *bulgaricus* milk.
D	0.19 lb.	0.45 lb.	0.21 lb.	0.44 lb.
F	0.12 lb.	0.15 lb.	0.18 lb.	0.20 lb.
G	0.12 lb.	0.12 lb.	0.16 lb.	0.17 lb.
Total	0.43 lb.	0.72 lb.	0.55 lb.	0.81 lb.

The total amount of ordinary sour milk consumed was 115.6 lbs., and that of *bulgaricus* milk 105.5 lbs. In spite of this difference the total amount of acids in the *bulgaricus* milk (1.53 lbs.) was far in excess of what the sour milk contained (0.98 lb.). If the value of sour milk feeding is dependent upon the amounts of acids, or the degree of acidity, present, the results obtained with the *bulgaricus* milk should have been far better than those following the sour milk diet. That this was not the case is clearly shown in the following condensed results (table VI).

TABLE VI.

Summary Showing the Comparative Influence of Sour Milk and of Bulgaricus Milk on Mortality.

Experiment.	No. of chicks.	Mortality.			
		Infected chicks		Uninfected chicks.	
		Fed sour milk.	Fed *bulgaricus* milk.	Fed sour milk.	Fed *bulgaricus* milk.
D	50	7, or 14%	10, or 20%	0	6, or 12%
F	53	18, or 34%	20, or 37.7%	6, or 11.3%	8, or 15.1%
G	45	25, or 55.5%	18, or 40%	11, or 24.4%	8, or 17.7%
Total	148	50, or 33.7%	48, or 32.4%	17, or 11.5%	22, or 14.8%

These figures require little comment. The total mortality of all chicks that received the naturally soured milk (both infected and uninfected) was 67, or 45.3 per cent., as compared with 70, or 47.3 per cent., for the *bulgaricus* chicks.

The small difference which is to the advantage of the sour milk feeding is, of course, within the limits of possible error in experiments of this kind, and should not be given much importance; nevertheless, the results clearly show that *bulgaricus* milk is of no greater value in reducing mortality than ordinary sour milk.

TABLE VII.

Figures Showing the Comparative Influence of Sour Milk and of Bulgaricus Milk on Growth (Experiments D, E, F, and G).

Average Gain per Ten Chicks.

Infected chicks.		Uninfected chicks.	
Fed sour milk.	Fed *bulgaricus* milk.	Fed sour milk.	Fed *bulgaricus* milk.
6.13 lbs.	5.61 lbs.	7.50 lbs.	6.19 lbs.

Combined averages for infected and uninfected chicks:

Fed sour milk ..... 6.82 lbs.
Fed *bulgaricus* milk ..... 5.90 lbs.
Difference ..... 0.92 lb.

These figures (table VII) are for experiments D, E, F, and G. If we eliminate experiment E because hens were used as brooders and no records could be made of the amounts of feed and milk consumed, the averages are as follows (table VIII).

TABLE VIII.

Average Gain per Ten Chicks in Experiments D, F, and G.

Infected chicks.		Uninfected chicks.	
Fed sour milk.	Fed *bulgaricus* milk.	Fed sour milk.	Fed *bulgaricus* milk.
5.48 lbs.	4.87 lbs.	7.1 lbs.	5.87 lbs.

Combined averages for infected and uninfected chicks:

Fed sour milk ..... 6.26 lbs.
Fed *bulgaricus* milk ..... 5.37 lbs.
Difference ..... 0.89 lb.

The pens that were supplied with the naturally soured milk (experiments D, F, and G) gained 0.89 of a lb. (or 14.2 per cent.) more per ten chicks than those which received the *bulgaricus* product. Since the former consumed more food (both dry feed and milk), but less acid, than the *bulgaricus* group, the greater gain in the sour milk pens must be attributed almost entirely to the food as such. The difference in gain per ten chicks closely corresponds with the difference in the total amount of solid food taken, namely 12.9 per cent., the sour milk chicks consuming 20.45 lbs. and the *bulgaricus* lots 17.81 lbs. Here again the differ-

ence is in favor of the naturally soured milk. Not only was there a larger absolute gain in weight in the sour milk chicks, but the gain per lb. of solid matter consumed was greater than in the *bulgaricus* lots. This difference has already been brought out in table II. The sour milk was the more appetizing, as 115.6 lbs. were consumed in experiments D, F, and G, while only 105.5 lbs. of the *bulgaricus* milk were utilized.

A COMPARATIVE STUDY OF THE INFLUENCE OF SWEET AND OF SOUR MILK ON MORTALITY AND GROWTH.

In the investigation of the past year (1914) eight complete experiments involving the use of 2,250 chicks were conducted.[6] The plan of these experiments was practically the same as the preceding.

Each lot of newly hatched chicks was divided into six uniform groups. Pens 1, 2, and 3 were artificially infected with 48 hour bouillon cultures of *B. pullorum*, while pens 4, 5, and 6 were left untreated. Pens 1 and 4 were fed ordinary well soured milk; pens 2 and 5 sweet milk, and pens 3 and 6 no milk at all. Thus, pens 4, 5, and 6 served as controls for infected pens 1, 2, and 3; and pens 3 and 6 were controls with no milk for pens 1, 2, 4, and 5. In all but two of the experiments the sweet milk was curdled with rennet tablets. The milk was supplied at least twice each day, usually early in the morning and at noon. In every case of milk feeding the milk was supplied as soon as the chicks were removed from the incubators, approximately twenty-four hours after hatching. In six of the experiments the chicks were artificially infected at the time that they first received the milk, as well as on each of the following four days. In the other two experiments the first infection was postponed until three or four days after the earliest milk feeding. In every instance the bouillon culture was administered five times on five consecutive days. The delaying of infection with *B. pullorum* was for the purpose of giving the chicks all the advantages of early milk feeding that it might possess, especially in as far as increasing bodily vigor is concerned.

The results of these experiments appear in the tables[7] under the following heads: numbers of chicks in each pen at the beginning of each week, weekly weights of the chicks, amounts of dry feed and of milk consumed per week as well as for the entire period, gains in weight per ten chicks for each pound of total solid matter, including milk consumed, and the mortality. The milk solids were estimated as 10 per cent. Table IX is a condensed statement of the data which bear on mortality.

[6] Rettger, Kirkpatrick, and Card. *loc. cit.*

[7] Rettger, Kirkpatrick, and Card, *loc. cit.*

TABLE IX.

Influence of Sweet and of Sour Milk Feeding on Mortality.

Experiment.	No. of chicks.	Mortality.					
		Infected chicks.			Uninfected chicks.		
		Fed sour milk.	Fed sweet milk.	No milk.	Fed sour milk.	Fed sweet milk.	No milk.
A	31	11	4	4	10	4	5
B	38	17	22	31	9	10	2
C	38	20	22	24	4	3	5
D	59	2	4	10	3	6	3
E	49	30	30	40	3	10	25
F	60	49	48	60	25	23	50
G	50	16	9	24	5	2	10
H	50	6	6	8	6	3	14
Total mortality, by pens		151, or 40%	145, or 39%	201, or 54%	65, or 17%	61, or 16%	114, or 30%

Total mortality for the three infected groups.....497, or 44.2 per cent.
Total mortality for the three uninfected groups...240, or 21.3 per cent.

The results obtained in experiments A and B were not as satisfactory and well defined as in the others. The chicks were hatched in March, and hence were kept indoors for the greater part of the time that they were under observation, the weather being cold. Considerable leg weakness developed, which was confined largely to the pens receiving milk. The sour milk pens in A were most seriously affected. More milk was consumed by these chicks than in the sweet milk lots. Furthermore, the conditions were more favorable for leg weakness at the time experiment A was in progress than later. It appeared most evident that the milk-fed chicks were growing too fast, under the conditions of close confinement, for the legs to support their weight.

The mortality in experiment F was unusually high in all the pens, especially in the uninfected lots. These figures alone constitute a large part of the total mortality. They are explained by the fact that the chicks used in this experiment were of low vitality, which was shown in various ways, aside from the high death rate. However, the proportion that the mortality figures of the milk-fed chicks bear to those which were not supplied with milk is about the same as the average for the eight experiments.

According to the above data there is a slight difference in the value of sweet and of sour milk feeding. Among the infected chicks the mortality was 2.5 per cent. greater in the sour milk than in the sweet milk pens, while among the uninfected the difference amounted to 5.9 per cent.

The value of milk feeding as such is clearly demonstrated in the

table. Not only was there a great reduction in the mortality of the uninfected lots that received milk (either sweet or sour), which amounted to almost 100 per cent., but there was a marked difference in the death rate of the milk-fed infected chicks, as compared with those which remained without milk, the difference amounting to approximately 30 per cent. In other words, there were almost twice as many deaths in the uninfected lots which were denied the milk than in the corresponding sweet or sour milk pens, and almost one-third more deaths in the infected groups that did not receive the milk than in the corresponding milk-fed lots. These results are in harmony with those of the first series of experiments (1912). Sufficient evidence is at hand, therefore, to show that the feeding of milk, either sweet or sour, exerts a most decided influence in lowering the general death rate of young chicks, and if fed soon enough greatly reduces the mortality from bacillary white diarrhea.

That the feeding of sour milk in the second series of investigations did not materially affect the death rate from bacillary white diarrhea may be explained readily by the fact that the milk was not supplied soon enough before the chicks were subjected to the artificial infection, and hence failed to increase the vigor and resistance of the chicks before the bacteria in question established themselves and the disease ran its natural course.

TABLE X.

Influence of Sweet and of Sour Milk Feeding on Growth.
Average Total Gain in Weight per Ten Chicks.

Infected chicks.			Uninfected chicks.		
Fed sour milk.	Fed sweet milk.	No milk.	Fed sour milk.	Fed sweet milk.	No milk.
4.62 lbs.	4.17 lbs.	2.65 lbs.	5.07 lbs.	4.71 lbs.	3.12 lbs.

Combined averages, all chicks:

Fed sour milk ..... 4.84 lbs.
Fed sweet milk ..... 4.44 lbs.
Fed no milk ..... 2.88 lbs.

TABLE X.—*Concluded.*

Gain in Weight per Ten Chicks for Each Pound of Total Solid Matter Consumed as Food.

	No. of chicks employed.	Fed sour milk.	Fed sweet milk.	No milk.
Infected	1,125	0.25 lb.	0.24 lb.	0.19 lb.
Uninfected	1,125	0.27 lb.	0.26 lb.	0.22 lb.
Combined (infected and uninfected)	2,250	0.26 lb.	0.25 lb.	0.205 lb.

In every instance (table X) the feeding of sweet and of sour milk was followed by a marked increase in the weights of the chicks beyond that which took place in the chicks which received no milk, the difference often amounting to as much as 80 per cent., and in two cases to more than 100 per cent. The differences vary in a large measure in direct proportion to the amounts of total solid matter consumed. Nevertheless, a review of the original tables[8] will show that, aside from its appetite-stimulating properties, the milk served to bring about a more complete utilization of the food.

The combined data on the 2,250 chicks show that those which received the sour milk gained 0.26 of a pound per ten chicks for each pound of total solids consumed; that those which were fed sweet milk made a gain of 0.25 of a pound; and those that were without milk gained only 0.20 of a pound. In other words, the milk-fed chicks gained 25 per cent. and 30 per cent. more in weight per pound of solid matter used than the chicks which received no milk. The gains per pound of solid food in the sour milk chicks were 5 per cent. greater than in the sweet milk lots. As this difference is so small as to lie within the limits of possible error, it should not be given too much importance. Furthermore, it offsets the slight difference in mortality that favored the sweet milk feeding (table IX).

The results of the entire investigation of milk feeding, which extended over a period of almost three years, fully justify the conclusion that milk is an important factor of diet in as far as mortality and growth are concerned. Whether milk is fed as sweet, ordinary sour, or *Bacillus bulgaricus* milk, the effect is essentially the same. Hence, milk as such possesses one or more ingredients which are of unique significance. No experiments have been undertaken, in connection

[8] Rettger, Kirkpatrick, and Card, *loc. cit.*

with the general problem of milk feeding, to determine what these physiologically active substances are. Some reference to the work of Osborne and Mendel will be of special interest here.

These investigators have shown that butter fat, when fed to rats which failed to complete their growth on a diet consisting of pure proteins, starch, protein-free milk, and commercial lard, enabled the rats to regain their lost growth.[9] They have also acquired an abundance of evidence to indicate that lactalbumin[10] is of particular value in fostering growth. In other words, lactalbumin furnished the necessary factors in their experiments which are required for normal growth. Since lactalbumin is rich in both tryptophane and lysine, it is but natural to assume that this milk protein owes its unique dietary properties to these two substances that it contains in its molecule. Osborne and Mendel have apparently demonstrated that tryptophane is necessary for maintenance, but wholly inadequate to produce growth, and that lysine is indispensable for growth.

II. THE INFLUENCE OF MILK FEEDING ON THE CHARACTER OF THE INTESTINAL FLORA.

That the value of the feeding of milk does not lie in acids that may be present in the milk, nor in acid-producing bacteria which as a rule constitute a large part of the organisms occurring in milk, has been clearly demonstrated in the foregoing investigations. These results are not in harmony with the ideas of many, who hold that sour milk, or the acid product which is prepared with the aid of the so called "Bulgara" or "Bulgaricus tablets," is of great dietary importance because of the presence of these acid-producing bacteria, or of their acid products.

After experimenting upon himself, Leva[11] arrived at the conclusion that the ingestion of Metchnikoff's lactobacilline brought about an acclimatization of *B. bulgaricus* in the intestine. This organism was found to be present in the feces on and after the fifth day following the use of the tablets. He also observed that during the investigation the amounts of aromatic oxyacids, hippuric acid, and phenol in the urine were decreased.

In an extensive investigation in which thirty different hospital patients were employed as subjects, besides the author himself, Cohendy[12] was led to conclude that the use of milk which is soured by *B. bulgaricus* causes a marked transformation of the intestinal flora, which is made apparent by the deodorization

[9] Osborne, Mendel, Ferry, and Wakeman, *loc. cit.*, 1913, xv, 311; 1913–14, xvi, 423; 1914, xvii, 401.

[10] Osborne, Mendel, Ferry, and Wakeman, *loc. cit.*, 1914, xvii, 325.

[11] Leva, J., *Berl. klin. Wchnschr.*, 1908, xlv, 922.

[12] Cohendy, M., *Compt. rend. Soc. de biol.*, 1906, lviii, 602.

of the feces and by a decrease in the amounts of conjugate sulphates in the urine. He ascribed the change to the lactic acid bacillus, without due regard to the milk itself.

Belonovsky[13] employed white mice. The diet consisted of sterilized wheat grain to which cultures of *B. bulgaricus* had been added. Twelve days after the beginning of the experiment a marked change was noted in the character of the intestinal flora. There was a large increase in the number of Gram-positive organisms, and a corresponding decrease in gas-producers. Similar results, although less pronounced, were obtained with mice that were fed sterilized wheat and killed cultures of *B. bulgaricus*. The author further states that in young nursing mice the intestinal flora presented practically the same microscopic appearance as in the older mice which received the wheat and *bulgaricus* cultures. Direct microscopic examination of the feces of the mice that received the *bulgaricus* bacillus cultures failed to reveal a predominance of the *bulgaricus* type over other organisms. In fact, it is to be inferred that bacilli of this description were very few. The writer believes that the action of *B. bulgaricus* is not due entirely to the bacilli, or to the lactic acid, but to other products as well.

The opinions cited above are essentially the same as those which Metchnikoff[14] announced and reiterated from time to time, and which have apparently gained a firm hold in the minds of many scientists as well as laymen. It is claimed that the wonderful accomplishments of *B. bulgaricus* are closely linked with its power to destroy or eliminate harmful bacteria from the intestinal tract, particularly those having putrefactive properties. Hence, by its ability to prevent autointoxication, it serves as an important therapeutic agent, even to the extent of preventing premature old age.

In the investigations upon which this part of the paper is based at least seventy-five white rats were employed. A moderate number of experiments was conducted also on chicks of different ages and on adult fowls. The work was the natural outcome of the earlier investigation of Rettger and Horton[15] on the intestinal flora of white rats kept on experimental and ordinary mixed diets.

Special emphasis has been given to organisms which belong to the type of *Bacillus bifidus* (Tissier) and of *Bacillus acidophilus* (Moro), though other types of intestinal organisms were not overlooked or neglected when they were present in appreciable numbers. *Bacillus acidophilus* assumed much prominence in the earlier feeding experiments which were carried on in connection with the elaborate investigations of Osborne and Mendel on the influence of pure pro-

[13] Belonovsky, J., *Ann. de l'Inst. Pasteur*, 1907, xxi, 991.

[14] Metchnikoff, E., Prolongation of Life, translated by Mitchell, P. C., London, 1907.

[15] Rettger, L. F., and Horton, G. D., *Centralbl. f. Bakteriol., 1te Abt., Orig.*, 1914, lxxiii, 362.

tein diets on growth. When the rats were transferred from the ordinary mixed to the pure protein diets a marked transformation in the character of the intestinal flora invariably took place. The flora became more simplified, very few types of bacteria being found after an interval of two or three days following the change of food. Organisms belonging to the *acidophilus* group always became numerous, and at times were so prominent as to exclude all other types except *Bacillus bifidus*. This change in the intestinal flora was undoubtedly independent of the pure proteins, but, as later experiments have indicated, bore a definite relation to the protein-free milk which formed a part of the pure protein diet.

The experiments[16] recorded here will be presented under three different heads; namely, the influence of milk feeding, the influence of carbohydrate feeding, and the influence of the ingestion of *Bacillus bulgaricus,* on the intestinal flora. The results of only a limited number of experiments which are typical will be given in detail.

The methods employed were essentially the same in the different series of experiments. The rats were kept in cages which were constructed of rather coarse wire net and supported at some distance over a tray which was covered daily with clean paper, so that the feces could be collected with as little chance of contamination as possible. Samples of feces were collected at regular intervals, sometimes daily. From 0.2 to 0.4 of a gram of the samples was vigorously shaken in test-tubes containing eight to ten cubic centimeters of sterile water and a little broken glass until a uniform suspension was obtained. From these suspensions agar plates and Veillon tubes were prepared in the desired dilutions. Smears were made also on slides and stained by the Gram method.

For plate-pouring different kinds of agar medium were used; namely, plain agar, dextrose agar (1 per cent. dextrose), acid dextrose agar (1 per cent. dextrose, 0.5 per cent. acetic acid), and neutral whey agar (neutral whey 1 liter, peptone 10 grams, agar 15 grams, and Liebig's meat extract 3 grams). The dextrose agar was used at practically all times, especially in the Veillon tubes.

[16] These experiments were conducted largely by Thomas G. Hull, and appear in part in another paper (Hull, T. G., and Rettger, L. F., *Centralbl. f. Bakteriol., 1te Abt., Orig.,* 1914, lxxv, 219).

All the rats were fed bread and green vegetables as the basic diet. In the different experiments the special agents to be studied were added to the basic diet. In several instances the bread was ground in a meat grinder. Numerous examinations of the feces of white rats that had been subsisting upon bread and green vegetable food for at least three or four days had shown that the intestinal flora were of the usual mixed type, the absence, or but a small number, of *Bacillus acidophilus* and *Bacillus bifidus* being particularly noticeable. Hence, such a diet was of much value in bringing the flora to a standard or uniform basis as a definite starting or end point in the different experiments.

MILK FEEDING.

In none of the experiments was the milk supplied until examination of the feces demonstrated that *Bacillus acidophilus* and *Bacillus bifidus* were absent from the feces, or that they were comparatively rare. Furthermore, control rats which received only the bread and vegetable diet were employed along with the others. The milk, which was usually whole milk, was poured over the dried bread crumbs or in the water dish in place of the water. The following experiments are given here as examples.

Experiment A.—Rats 1, 2, and 3 received 20 to 30 c.c. of milk three times a week for four weeks, in addition to the basic diet, while rats 4, 5, and 6 were given the bread and vegetable food only. During the entire period *B. acidophilus* was practically absent from the feces of the control rats, whereas in the milk-fed rats it was abundant after the first few days and continued so until the milk feeding was discontinued, after which the *acidophilus* group again disappeared.

Experiment B.—Rats 2 and 3 received, in addition to the bread and vegetable diet, 10 c.c. of milk daily for ten days. After the first three days *B. acidophilus* appeared, and numerous colonies of this organism were obtained on the agar plates. It was also very abundant in smears prepared from the feces, although various other types were present in small numbers.

Experiment C.—Rat 23 was supplied with 50 c.c. of milk daily for forty days. Within two to three days *B. acidophilus* became very numerous, which was shown by the large numbers of colonies on the agar plates, and by the small number of other types, excepting *B. bifidus,* in the microscopic mounts. Besides *B. acidophilus,* the Gram stained smears contained numerous rods which were smaller than the others, and which showed evidence of branching (*B. bifidus*). In the Veillon tubes *B. acidophilus* was quite abundant throughout the length of the tubes in the form of the typical shaggy colonies, while the smooth, disc-like colonies of *B. bifidus* appeared in sharp contrast in the deeper portions of the

tubes. As the experiment progressed *B. bifidus* increased in numbers, and in a large measure supplanted *B. acidophilus*.

At the termination of the forty days' period but little milk was taken by the rat, and although milk was supplied for the next ten days very little was consumed. As a consequence, *B. bifidus* disappeared from the intestine, and the number of *B. acidophilus* was greatly reduced, while other types again became more common.

Postmortem examinations were made of rats 2 and 3 after they had been on the bread, vegetable, and milk diet for ten days. Cultural and microscopic tests of the gastro-intestinal tract revealed the presence of *B. acidophilus* in large numbers, and in almost pure form, in the small intestine. It was also found to be abundant in the large intestine, but here it was mixed with various other organisms.

Numerous other experiments on milk feeding, both with white rats and with chicks of different ages, have given similar results. Sufficient evidence is at hand, therefore, to show that profound alterations in the character of the intestinal flora, at least of the white rat and the common domestic fowl, are brought about by the addition of milk to a diet which has established a flora in which the *acidophilus* and *bifidus* types of bacteria are few or absent. In this transformation the usual mixed flora are, in a large measure at least, supplanted by ones rich in *Bacillus acidophilus* or *Bacillus bifidus*, or both of these organisms. A limited number of experiments on man have given results similar to the above. The complete transformation of types is, however, not accomplished so readily as in the rat or in fowls.

Since *Bacillus acidophilus* and *Bacillus bifidus* are both carbohydrate-consuming organisms, and have but slight proteolytic properties, it seemed but natural to look for a possible explanation of the above results in the lactose content of the milk. For this purpose numerous feeding experiments were conducted on white rats with seven different carbohydrates.

CARBOHYDRATE FEEDING.

The following carbohydrates were used: lactose, sucrose, maltose, dextrose, levulose, galactose, and dextrine. At first each carbohydrate was fed in the form of a concentrated aqueous solution, which was poured over the bread crumbs; later three to four grams of the carbohydrate in question were placed on the bread and moistened

with a small amount of water. The lactose feeding experiments were carried on for periods varying from one to seven weeks.

Experiment A.—Rat 13 was fed lactose for fifteen days. On the third day *B. acidophilus* was observed in appreciable numbers in the smears and agar plates prepared from the feces, after which it practically disappeared, and *B. bifidus* was found to be present in almost pure form in the smears.

Experiment B.—Rat 12 received lactose for thirty days, but little change was observed in the intestinal flora, neither *B. acidophilus* nor *B. bifidus* being present in appreciable numbers in the slides, plates, or Veillon tubes. No explanation can be given for this irregularity in the results, except that the rat was peculiarly resistant to bacterial changes in the intestine, which as a rule are readily induced by change of diet.

Experiment C.—Rats 16 and 17 obtained milk sugar for eleven days. *B. bifidus* became quite prominent on the second day and continued to the end of the period. *B. acidophilus* also was present in small numbers.

Experiment D.—Rat 35 was given lactose for thirteen days. *B. acidophilus* became very abundant at the outset, but soon diminished in numbers, while *B. bifidus* increased to such an extent as to exclude almost all other forms.

Experiment E.—Rat 26 received lactose for fifty days. For a short time the intestinal flora consisted largely of *B. bifidus* and, to a lesser extent, of *B. acidophilus.* Later *B. bifidus* was practically the only organism present.

Experiment F.—Rats 21 and 22 were fed milk sugar for sixteen days; Nos. 14, 15, and 16 for twenty days; Nos. 17 and 19 for eight days; Nos. 23, 27, and 28 for seven days, and No. 9 for ten days. In every instance *B. bifidus* became very abundant soon after the addition of the lactose to the diet. In general, the following changes took place in the intestine. By the second or third day *B. acidophilus* and *B. bifidus* began to make their appearance. After having attained a certain maximum *B. acidophilus* gradually decreased in number, and was practically absent at the close of the period, with very few exceptions. *B. bifidus* increased very fast, however, and in every case it became the predominant form.

Several lactose rats were killed for the purpose of making bacteriological examinations of the digestive tract. In rat 9 no colonies of *B. acidophilus* or *B. bifidus* were obtained from the stomach. A small number of the *acidophilus* type was found in the duodenum, while throughout the remainder of the intestine *B. bifidus* was quite abundant. In rat 26 (fed lactose for fifty days) *B. bifidus* was present in large numbers in the stomach, the small intestine below the duodenum, and in the large intestine. *B. acidophilus* was found also, but to a limited extent. Rat 15, which required an unusual length of time for *B. bifidus* to establish itself, contained *B. acidophilus* in the stomach and throughout the small and large intestine, while *B. bifidus* was plentiful in the ilium and in the large intestine.

The foregoing experiments demonstrate that both milk and lactose, when fed in sufficient quantities, have a pronounced influence on

the character of the intestinal flora, each bringing about a profound change in which the ordinary mixed flora give way to ones that are more simplified and which are made up largely of *Bacillus acidophilus* or *Bacillus bifidus,* or both. That milk owes this property to the lactose which it contains can hardly be doubted. Since almost half of the total solids of milk is milk sugar, namely, about 5 per cent., it is not surprising that milk when fed even in small amounts has such a marked influence on the flora of the intestine.

A large number of feeding experiments was conducted in which sucrose, maltose, dextrose, levulose, galactose, and dextrine were used in their turn. Very little or no change could be observed in the intestinal flora of any of the rats. In order to determine whether the negative results were due to an inhibiting influence of the different carbohydrates, a limited number of feeding experiments was carried out in the following manner. Rats which had been receiving lactose, and which exhibited the characteristic flora of milk- or lactose-fed rats, were given, besides the usual amount of milk sugar (three to four grams daily), an equal amount of the carbohydrate in question. No decrease could be observed in the numbers of organisms of the *acidophilus* and *bifidus* types, nor was there any other apparent change. The failure, therefore, of the intestinal bacteria to be affected by any of these carbohydrates, except lactose, can not be ascribed to any inhibitory action which they or their products may exert.

Thus far one significant fact has been demonstrated repeatedly; namely, that diet may be a very important factor in determining the character of the intestinal flora.

The above results are in accord with those of Sittler,[17] who found that when lactose is fed to children that subsist on cow's milk the typical *bifidus* flora of breast-fed infants are present. Similar results were obtained with so called "malt soup." On the other hand, cane sugar did not react in this way. The limited number of experiments which was carried on with dextrose seemed to indicate that it played the same part as lactose. This claim could not be substantiated by us.

Quite recently Distaso and Schiller[18] claim to have shown that the feeding of milk, lactose, or dextrine to white rats which have been on a diet of bread and meat brings about a change in the intestinal flora from the usual mixed to

[17] Sittler, P., *Centralbl. f. Bakteriol., 1te Abt., Orig.,* 1908, xlvii, 14.

[18] Distaso, A., and Schiller, J., *Compt. rend. Soc. de biol.,* 1914, lxxvi, 243.

the *bifidus* type. No mention in their brief publication is made of *B. acidophilus.* Our results on the feeding of dextrine are not in harmony with those just cited. The difference may be due to the dextrine employed. Further work to determine the influence of dextrine, and starches as well, is under way.

It is surprising that so little attention has been given in past investigations to the occurrence of *B. acidophilus* (Moro),[19] and to the conditions that influence its numbers in the intestine. In our work on the intestinal flora of the albino rat and of the common domestic fowl, organisms of the *acidophilus* type have been met with almost constantly. In the feces of man and of guinea pigs we have found them frequently also, and at times in considerable numbers. While *B. bifidus* (Tissier)[20] was in most instances associated with *B. acidophilus,* the latter was, as a rule, much more prominent. Exceptions to this rule occurred in some of the lactose feeding experiments.

THE INFLUENCE OF THE INGESTION OF BACILLUS BULGARICUS ON THE CHARACTER OF THE INTESTINAL FLORA.

The feeding of *Bacillus bulgaricus* was accomplished in two ways. In some instances the rats received milk cultures of the organism. These cultures were prepared by inoculating sterilized skimmed milk with the organism and incubating for twenty-four hours at 37° C. By the other method large surfaces of dextrose agar were inoculated with *Bacillus bulgaricus.* After an incubation period of twenty-four hours the surface growth was washed off with sterile water. The water suspensions were fed as such in small dishes, or they were poured over the bread crumbs. In all the *bulgaricus* feeding the regular basic diet of bread and vegetables was employed. In some of the experiments sterile milk was fed to one or two of the rats, instead of the inoculated milk or the bacterial suspensions, also the washings of sterile agar.

It was difficult at the outset to distinguish between *Bacillus bulgaricus* and *Bacillus acidophilus,* and especially the colonies on dextrose agar. Certain marks of distinction were noted, however, but the identification was never regarded as satisfactory without the milk acidification test. Since *Bacillus bulgaricus* rapidly produces enough acid to coagulate the milk, and a recently isolated *acidophilus* strain does so only after more prolonged incubation, this test was of considerable importance.

Special precautions had to be taken to minimize the chances of

[19] Moro, E., *Jahrb. f. Kinderheilk.,* 1900, lii, 38.

[20] Tissier, H., *Ann. de l'Inst. Pasteur,* 1905, xix, 109; 1908, xxii, 189.

contamination of the feces by *bulgaricus* bacilli that were supplied with the food. This was accomplished with no little effort.

Experiment A.—Six rats were employed. Rats 1 and 4 received the basic diet of bread and vegetables together with a watery suspension of *B. bulgaricus;* rats 2 and 5 were given the washings of sterile agar, in addition to the regular diet, while Nos. 3 and 6 received the bread and vegetable only. The experiment continued for four weeks, with daily feeding. *B. bulgaricus* was observed but twice in the feces of the rats that were abundantly supplied with it. Furthermore, no differences could be detected between the flora of rats 1 and 4 and those of the four rats which were not supplied with the organism in question. In all the rats the typical mixed flora prevailed.

In postmortem examinations conducted on rats 1 and 4 eighteen hours after the last feeding of *B. bulgaricus,* an organism resembling the *bulgaricus* bacillus was found in very small numbers in the stomach and duodenum, while from the remainder of the intestine not a single colony of this organism could be obtained.

Experiment B.—Rat 21 received the stock diet together with a washed culture (water suspension) of *B. bulgaricus;* No. 22 was given the regular diet plus 50 c.c. of milk that was fermented by *B. bulgaricus,* and rat 23 the usual diet plus 50 c.c. of sterilized milk. From the feces of rat 21 single colonies resembling those of *B. bulgaricus* were obtained three times; from No. 22 individual colonies of the *bulgaricus* type were obtained but four times, while in the plates from rat 23 no colonies of *B. bulgaricus* were detected, though colonies of *B. acidophilus* were quite abundant. This experiment continued over a period of four weeks.

Experiment C.—Rat 13 received bread and lettuce plus the water suspension of *B. bulgaricus;* No. 32 was given the same diet plus 50 c.c. of sterile milk and the water suspension; and rat 23 the same basic diet plus 50 c.c. of sterile milk. *B. bulgaricus* could at no time be found in the feces of the rats (13 and 32) that were fed the living bacilli; nor was there any apparent difference between the intestinal flora of rats 32 and 23. This experiment lasted ten days.

On postmortem examination one of the three rats that were given *B. bulgaricus* in water suspension failed to show the presence of this organism in any portion of the digestive tract; in the second a very small number was recovered from the jejunum and ilium only. The third rat had been receiving lactose for some time. *B. bulgaricus* could not be recovered from it, while *B. bifidus* was found in large numbers.

In all the rats that received milk, whether it was sterile or inoculated with *B. bulgaricus, B. acidophilus* and *B. bifidus* were at all times more or less prominent, as in the previous milk-feeding experiments. On the other hand, in spite of the large numbers of *bulgaricus* bacilli that were fed to some of the rats, very few could be recovered from the feces, and at no time did this organism establish itself in the intestine, even after the daily ingestion of the bacilli for periods of from ten days to four weeks.

These observations are in accord with those of Luerssen and Kühn,[21] who failed to establish *B. bulgaricus* in the intestine of man by the continued use of

[21] Luerssen, A., and Kühn, M., *Centralbl. f. Bakteriol., 2te Abt.,* 1908, xx, 234.

yoghurt, and with the views of Distaso and Schiller, [22] who claim to have shown quite recently that the ingestion of large numbers of this organism by rats which are receiving milk or lactose does not result in its acclimatization in the intestine, and that *B. bifidus* is the predominating type of bacteria.

Oehler[23] conducted some feeding experiments with yoghurt on mice and monkeys, and stated that the *bulgaricus* bacillus could be demonstrated with ease in the feces during the yoghurt feeding period. Within two to three days after the yoghurt feeding was discontinued, *B. bulgaricus* again disappeared from the intestine. It should be borne in mind that large quantities of milk as such were supplied in the form of the yoghurt, and hence the conditions for the development of *B. acidophilus* were most favorable. Some doubt may be felt, therefore, as to whether Oehler's *B. bulgaricus* may not have been the Moro bacillus. It is conceivable, however, that any species of organism may be recovered in limited numbers from the feces if ingested in extremely large numbers.

Herter and Kendall[24] found that in a monkey which was killed three to four hours after it had been fed 500 c.c. of milk that had been well soured by bacillac very few *bulgaricus* bacilli were present in the large intestine. In an earlier experiment on this monkey they had failed to detect *B. bulgaricus* in the feces after feeding the sour milk (bacillac) daily for three days. These same authors state that the intestinal flora of cats and monkeys were rapidly altered when a diet of meat or eggs was followed by one of milk and dextrose. The most important change was the substitution of an acidophilic, non-proteolyzing type of flora for one which was strongly proteolytic.

Even *B. coli*, especially a foreign strain, appears to be unable to establish itself in the intestine as the result of the feeding of this organism. This was clearly demonstrated by Rettger and Horton[25] who supplied white rats which were on diets containing pure protein as the only available nitrogenous food with vast numbers of colon bacilli. In one of the experiments a very small increase of *B. coli* took place in the intestine; while in another there was no perceptible increase, although *B. coli* was fed at frequent intervals for a period of almost four weeks.

Similar results were obtained by Seiffert[26] who showed that a strain of *B. coli* isolated from another species failed to inure itself to the conditions which obtain in the human intestine after being taken into the system *per os*. A strain which had been isolated from the same person, however, rapidly multiplied and was found in abundance in the intestine. Raubitschek[27] also claims to have demonstrated that foreign organisms are unable to establish themselves in the intestine, except after at least partial immunization of the host to the particular organisms.

Kulka[28] conducted a series of experiments on man and rabbits. His results appear to be very decisive, demonstrating that *B. metchnikovi* and *B. prodigiosus*,

[22] Distaso and Schiller, *loc. cit.*

[23] Oehler, R., *Centralbl. f. Bakteriol., 2te Abt.*, 1911, xxx, 149.

[24] Herter, C. A., and Kendall, A. I., *Jour. Biol. Chem.*, 1908–9, v, 293.

[25] Rettger and Horton, *loc. cit.*

[26] Seiffert, G., *Deutsch. med. Wchnschr.*, 1911, xxxvii, 1064.

[27] Raubitschek, H., *Virchows Arch. f. path. Anat.*, 1912, ccix, 209.

[28] Kulka, W., *Arch. f. Hyg.*, 1914, lxxxii, 337.

hen introduced either by mouth or by subcutaneous, intravenous, or intra-eritoneal injection, do not appear in the feces. Mitchell and Bloomer[29] ob-ined similar results in the common domestic fowl with the typhoid bacillus.

GENERAL CONCLUSIONS.

Throughout the investigations upon which a large part of this aper is based the favorable influence of milk feeding on mortality nd growth was most apparent. Mortality from all causes was requently reduced to at least one-half of what obtained among the hicks that received no milk, while the milk-fed chicks in some ex-eriments gained twice as much in weight as those that were without his article of diet. Although the influence of milk feeding was less ronounced on the mortality of chicks that were artificially infected vith *Bacterium pullorum,* quite an appreciable difference in mor-ality was always noted if the milk was fed at least one or two days efore the first administration of the bouillon cultures of the organ-sm in question.

Practically the same results were obtained, whether sweet or sour nilk was fed, and no differences could be observed in the relative alue of ordinary sour milk and of the so called *bulgaricus* product. Hence, the unique properties of this food exist in the milk as such, ather than in any milk acids or milk bacteria that may be present.

Milk and lactose diet exert a very important influence on the char-cter of the intestinal bacteria, especially in white rats and in the ommon domestic fowl. Within a few days after the ingestion of nilk or lactose a transformation of the flora takes place in which the sual mixed bacterial flora give way to ones that are more simplified, nd in which *Bacillus acidophilus* and *Bacillus bifidus* are, as a rule, rominent. It is to be assumed that milk has this influence in virtue f the large amount of lactose which it contains. Other carbohy-lrates, besides milk sugar, failed to bring about such a transforma-ion.

The ingestion of foreign bacteria, even in large numbers, does ot of itself bring about an elimination or displacement of the com-non intestinal microörganisms. Vastly more important is the influ-nce of diet, especially milk and lactose. The feeding of Bulgara

[29] Mitchell, O. W. H., and Bloomer, G., *Jour. Med. Research,* 1914, xxxi, 247.

tablets or other preparations which contain as the supposedly active agent the bacillus of Metchnikoff and Mazé, without due regard to the use of milk, can, therefore, be of little, if indeed of any, value. The beneficial effects which it is claimed have been derived from the use of yoghurt, and other oriental sour milk products have in all probability been due to the milk as such, rather than to the bacteria which they contained. This view is strongly supported by the extensive milk feeding experiments on chicks which are recorded in this paper, and also by the results which show the influence of milk and of lactose feeding on the intestinal flora of white rats and of the common domestic fowl.

CONCENTRATION OF THE PROTECTIVE BODIES IN ANTIPNEUMOCOCCUS SERUM BY MEANS OF SPECIFIC PRECIPITATION.*

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Previous work has shown that the occurrence of a specific precipitin reaction between a serum of one animal species and its antiserum may result in a very definite action on normal or immune antibodies contained in the first (precipitinogenic) serum.

Such studies were inspired by the work of Camus and Gley (1), Kossel (2), and Bordet (3), who had shown that antihemolysins were formed by immunization against a hemolytic serum, and were designed to determine whether similar antibodies could be produced to antitoxins, agglutinins, precipitins, and the like.

Kraus and Eisenberg (4) succeeded in producing an antilactoserum, the action of which was apparently due to the carrying down of the precipitin of the lactoserum with its precipitinogen which had been allowed to interact with the antilactoserum. In other words, the serum of a rabbit immunized against milk when precipitated by the serum of a goat immunized against rabbit serum removes the precipitin for milk from the lactoserum. The first combination was for some reason not effective when a dog-antirabbit serum was employed. These same authors failed, however, to demonstrate fixation of tetanus antitoxin or of typhoid agglutinin in horse serum after precipitating the horse serum by means of rabbit-antihorse serum. Their failure to remove the antitoxin was, however, due to the excess of precipitinogen (horse antitoxin) employed, an inhibiting factor to precipitate formation that was then unknown. This difficulty was overcome by Dehne and Hamburger (5), who found that diluted (1:500) horse antitoxin was entirely removed from the supernatant fluid on producing a precipitate with rabbit-antihorse serum. These experiments were fully corroborated by Kraus and Pribram (6), Hamburger (7), and von Eisler and Tsuru (8), and analogous facts were produced in respect to the fixation of diphtheria antitoxin in horse serum by Weill-Hallé and Lemaire (9) and by Atkinson and Banzhaf (10). Experiments which indicate that a similar removal of antitoxin takes place in the body of immunized animals were performed with diphtheria antitoxin by Sacharoff (11) and with tetanus antitoxin by Dehne and Hamburger (5).

Some difference of opinion exists as to the carrying down of agglutinins with a precipitinogen when antiserum is added, the results obtained doubtless depending on technical differences. Thus, Kraus and Eisenberg (4) at first failed to remove immune agglutinins, but later Kraus and Pribram (6) succeeded. Von Eisler and Tsuru (8) and Landsteiner and Prašek (12) brought down normal

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agglutinins, but the latter at times failed with immune agglutinins. Wassermann and Bruck (13) failed to remove antityphoid protective bodies by precipitation with rabbit-antigoat serum, whereas some such removal is indicated by the experiments of Walker (14) performed *in vivo*. Zebrowski (15) failed to remove the hemolytic sensitizer (amboceptor) by similar treatment.

These facts are of significance in considering the mode of action of antisensitizers (antiamboceptors) as bearing on the relations of lysins to "Bordet's fixation bodies," and particularly as bearing on the relation of the fixation reaction to the formation of specific precipitates (16). We do not propose, however, to discuss any of these interesting and somewhat puzzling questions at this time, but to direct attention to the one fact that an antiserum (antitoxin, agglutinating serum) when employed as a precipitinogen by the addition of an homologous antiserum (precipitin) may yield to the precipitate its entire content of antibody.

It is the reverse of this reaction which we wish to describe, employing antipneumococcus serum as precipitin and producing a precipitate by the addition of an extract of the pneumococcus. We find that such a precipitate brings down more or less completely the antibodies which protect animals against pneumococcus infection. Owing to a corresponding reversal of the relative dosage of the reacting substances, this reaction becomes at once of practical as well as of theoretical interest. It enables us not only to concentrate the specific antibodies of the antipneumococcus serum in small volume, but, as we shall see, in a fluid of extremely low protein content.

Although bacterial precipitins were the first to be described (Kraus, 1897 (17)), interest in them has for practical reasons been far exceeded by that taken in the serum and protein precipitins later described by Tchistovitch (18) and by Bordet (19). Neufeld (20) first described a precipitin for pneumococcus extract in the serum of specifically immunized animals. His observations were extended by Wadsworth (21), by Panichi (22), by Heyrovský (23), and by Norris (24).

In our own work we have employed potent antipneumococcus sera produced in horses by prolonged treatment with dead and living cultures of either type I or type II (25) strains of pneumococcus. Such sera agglutinate specifically the type of pneumococcus employed for

immunization in low dilutions, but have no effect on other types. They further protect mice in a dose of 0.2 of a cubic centimeter against 0.1, or rarely 0.2, of a cubic centimeter of a twenty-four hour bouillon passage culture of pneumococcus of which 0.000,001 of a cubic centimeter suffices to kill controls.

As antigen (precipitinogen) we have used water-clear extracts of dried and ground pneumococci obtained by precipitating considerable amounts of washed concentrated bouillon cultures by the addition of equal parts of 95 per cent. alcohol, or, in later experiments, by adding them to ten volumes of acetone (26) (Kahlbaum's C. P.).[1] These extracts were made in the manner that Gay and Claypole (27) have described for producing plain typhoid vaccines, and are made by suspending weighed amounts of ground culture in carbolated (0.5 per cent.) saline solution in the proportion of one milligram to one cubic centimeter, shaking at intervals at a temperature of 37° C. for from three to six hours, and then allowing them to remain in the ice box over night. On the following day the suspended mixture is vigorously centrifugalized until the supernatant fluid is cloudless and water-clear, or at most slightly opalescent. Such supernatant fluids from ground pneumococci are very toxic for mice, distinctly more so than the bacterial sediment from which they have been separated.

This clear antigen gives a distinct precipitin reaction with one cubic centimeter of strong immune serum in a dose of 0.01 of a cubic centimeter or less, when read from the sediment after twenty-four hours at room temperature. The maximal precipitate and rapidity of reaction are produced by the addition of from 0.3 to one cubic centimeter of the antigen when added in a single dose. We have as yet no information at hand as to the zone of inhibition or resolution of the precipitate in an excess of antigen. In producing precipitates to test for protective purposes our method has been to endeavor to produce the maximum precipitate from a given amount of immune serum. For this purpose we have added the antigen in divided doses, allowing the cloudy or flocculent precipitate to form for from a few minutes to several hours, centrifugalizing, and then

[1] This was done at the suggestion of Dr. Van Slyke.

adding more precipitinogen to the supernatant serum until no further cloud is produced.

We have by no means fully determined the ideal conditions for producing the maximal precipitate, or perhaps, more correctly speaking, the optimal protein complex in a precipitate, as is indicated by the considerable variations in the total nitrogen determinations of our washed precipitates. Nor have we in all instances succeeded in bringing down all the protective antibodies in our precipitates. The fact, however, that we have repeatedly found such precipitates, washed free of serum, to contain as much protective value in aliquot parts as the immune serum, seems to us to justify this communication, pending a greater uniformity of results.

EXPERIMENTAL.

Experiment 1.—To 20 c.c. of antipneumococcus serum (type I, lot 14, Dec. 6, 1913) were added 6 c.c. of clear carbolated extract of dried pneumococci. An immediate voluminous precipitate occurred. This was centrifugalized five minutes later and another 6 c.c. of antigen were added to the supernatant fluid. A slow cloudiness appeared which was allowed to form for two hours at 37° C. The second supernatant fluid in a dose of 1 c.c. gave no further precipitate with doses of from 0.1 to 1 c.c. of antigen. The combined precipitates were shaken in 20 c.c. of carbolated saline, centrifugalized, and resuspended. 20 c.c. of the same specimen of antipneumococcus serum were diluted *pari passu* with the above by addition of two doses of 6 c.c. each of carbolated saline solution instead of antigen.

One-half the above washed precipitate was dissolved by 2 c.c. of $\frac{N}{10}$ sodium hydrate. It was further diluted with saline until 0.5 c.c. of the solution corresponded to the precipitate derived from 0.2 c.c. of original serum. The undissolved precipitate, the original diluted serum, and the exhausted (precipitin-free) serum were also made up to corresponding volume (0.5 c.c. equals 0.2 c.c. of original serum).

A Kjeldahl determination by Mr. Cullen gave a total nitrogen in the undissolved precipitate, indicating a protein content of 0.34 per cent.

With these mixtures the following experiments were performed on mice (tables I, II, and III).

TABLE I.

A. Protective Experiment with Serum and Derivatives Mixed in a Fixed Amount with Decreasing Amounts of Dilutions of a Twenty-Four Hour Bouillon Culture of Pneumococcus, Type I, 96–1,[2] in a Total Volume of 0.5 C.C. The Mixtures Were Injected Intraperitoneally in Mice.

Mouse.	Protective fluid.	Culture.	Result.
1	Control	0.0001 c.c.	Died, 45 hrs.
2	Control	0.00001 c.c.	Died, 45 hrs.
3	Control	0.000001 c.c.	Died, 45 hrs.
4	Original serum 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
5	Original serum 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
6	Original serum 0.2 in 0.5 c.c.	0.01 c.c.	Died, 34 hrs.
7	Original serum 0.2 in 0.5 c.c.	0.001 c.c.	Survived.
8	Exhausted serum 0.2 in 0.5 c.c.	0.5 c.c.	Died, 20 hrs.
9	Exhausted serum 0.2 in 0.5 c.c.	0.1 c.c.	Died, 45 hrs.
10	Exhausted serum 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
11	Exhausted serum 0.2 in 0.5 c.c.	0.001 c.c.	Survived.
12	Whole washed precipitate 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
13	Whole washed precipitate 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
14	Whole washed precipitate 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
15	Whole washed precipitate 0.2 in 0.5 c.c.	0.001 c.c.	Died, 45 hrs.
16	Dissolved precipitate 0.2 in 0.5 c.c.	0.5 c.c.	Died, 45 hrs.
17	Dissolved precipitate 0.2 in 0.5 c.c.	0.1 c.c.	Survived.
18	Dissolved precipitate 0.2 in 0.5 c.c.	0.01 c.c.	Survived.
19	Dissolved precipitate 0.2 in 0.5 c.c.	0.001 c.c.	Died, 4 dys.

TABLE II.

B. Protective Experiment with Fixed Dose of Culture and Decreasing Doses of Serum and Serum Derivatives.

Mouse.	Protective fluid. Total volume 0.5 c.c.	Culture. Pneumococcus, type I, 98–1.	Result.
1	Original serum 0.1 c.c.	0.05 c.c.	Survived.
2	Original serum 0.05 c.c.	0.05 c.c.	Survived.
3	Original serum 0.025 c.c.	0.05 c.c.	Survived.
4	Original serum 0.0125 c.c.	0.05 c.c.	Survived.
5	Original serum 0.00625 c.c.	0.05 c.c.	Died, 14 dys.
6	Dissolved precipitate 0.1 c.c.	0.05 c.c.	Survived.
7	Dissolved precipitate 0.05 c.c.	0.05 c.c.	Survived.
8	Dissolved precipitate 0.025 c.c.	0.05 c.c.	Survived.
9	Dissolved precipitate 0.0125 c.c.	0.05 c.c.	Survived.
10	Dissolved precipitate 0.00625 c.c.	0.05 c.c.	Survived.
11	Exhausted serum 0.1 c.c.	0.05 c.c.	Survived.
12	Exhausted serum 0.05 c.c.	0.05 c.c.	Died, 36 hrs.
13	Exhausted serum 0.025 c.c.	0.05 c.c.	Died, 36 hrs.
14	Control	0.05 c.c.	Died, 20 hrs.
15	Control	0.0001 c.c.	Died, 18 hrs.
16	Control	0.00001 c.c.	Died, 40 hrs.
17	Control	0.000001 c.c.	Died, 48 hrs.

[2] This indicates the ninety-sixth passage through animals with a single passage on culture media.

TABLE III.

C. Duration of Protection Afforded by Antipneumococcus Serum and Its Precipitate.

In this experiment two series of mice were given, respectively, whole serum and dissolved precipitate in a dose of 0.2 c.c. subcutaneously, and inoculated subsequently at intervals with suitable controls.

Series *A*, on Dec. 15, 1914, was given 0.2 c.c. of diluted serum in saline (volume 0.5 c.c.) subcutaneously.

Series *B*, on the same date, was given 0.2 c.c. of dissolved precipitate from serum (0.2 in 0.5 c.c.) subcutaneously.

Mouse.	Interval.	Dose of culture.	Result.
A–1	4 dys.	0.1 c.c.	Died, 14 hrs.
A–2	4 dys.	0.01 c.c.	Died, 18 hrs.
A–3	4 dys.	0.001 c.c.[3]	Survived.
A–4	8 dys.	0.01 c.c.	Died, 20 hrs.
A–5	8 dys.	0.001 c.c.	Died, 10 hrs.
A–6	8 dys.	0.0001 c.c.[4]	Survived.
A–7	10 dys.	0.0001 c.c.	Survived.
A–8	10 dys.	0.00001 c.c.[5]	Survived.
B–1	4 dys.	0.1 c.c.	Died, 18 hrs.
B–2	4 dys.	0.01 c.c.	Died, 18 hrs.
B–3	4 dys.	0.001 c.c.[3]	Survived.
B–4	8 dys.	0.01 c.c.	Died, 10 hrs.
B–5	8 dys.	0.001 c.c.	Died, 10 hrs.
B–6	8 dys.	0.0001 c.c.[4]	Died, 4 dys.
B–7	10 dys.	0.001 c.c.	Died, 24 hrs.
B–8	10 dys.	0.0001 c.c.	Died, 30 hrs.
B–9	10 dys.	0.00001 c.c.[5]	Died, 42 hrs.

The first two sections of this experiment (*A* and *B*) show that the precipitate derived by adding an extract of pneumococcus to antipneumococcus serum may contain as much protective power against pneumococcus infection in mice as the original serum from which it is derived. It is active whether employed in its original precipitated condition or dissolved in a small amount of alkali. In this experiment the duration of the immunity passively conferred by precipitate was not so great as when serum was used. This failure in durability, however, may well have been due to the fact that the solution of

[3] Controls with doses of 0.00001 and 0.000001 c.c. died in 40 hrs.

[4] Controls with doses of 0.00001 and 0.000001 c.c. died in 20 and 48 hrs.

[5] Controls with doses of 0.00001 c.c. died in 42 hrs.

precipitate in an alkali is by no means without harmful effect on its protective power. The amount of alkali needed to dissolve the precipitate has varied not only with the apparent opacity of the precipitate but also with its age, freshly formed precipitates being more readily soluble than older ones. At all events the addition of the alkali has frequently robbed the precipitate of its protective properties, and it has likewise a harmful effect on the original serum. A solution of the precipitate seemed desirable in view of its possible application by intravenous injection in human cases of pneumonia. It is questionable, however, if the precipitate itself would be dangerous to employ; we have given considerable amounts to rabbits intravenously without ill effect.

It seemed to be of importance, when employing the original precipitate in protection against intraperitoneal infection in mice, to show that the protective effect is not in some measure due to a possible mechanical effect of the precipitate rather than to specific antibodies. This would seem to be ruled out in the following experiment, in which a precipitate from a serum of type I was employed mixed with cultures of pneumococci of both type I and type II.

Experiment 2.—Protective value of precipitate I.[6] The dried culture in this experiment differs from the preceding antigen in that it was precipitated by alcohol directly from the bouillon culture without washing. An extract was made in carbolated saline in dilution of 1 mgm. to 1 c.c. This antigen solution was added in doses of 50 and 40 c.c., with 12 hours' interval, to 100 c.c. of serum of type I, lot 3. Precipitates were produced on each addition, which were then washed and suspended in one-fifth the original volume. Not all the precipitate was removed from the treated serum, as could be shown by further addition of antigen. A Kjeldahl determination of the precipitate diluted to original volume gave a protein content of 0.108 per cent.

The protective values of the precipitate concentrated five times, of the precipitate at original volume, and of the concentrated precipitate dissolved in sodium hydrate,[6] are compared with the original serum. Mixtures of the various fluids were made with the culture dilutions, each in a volume of 0.5 c.c., and injected intraperitoneally in mice (table IV).

[6] 0.3 c.c. of $\frac{N}{1}$ sodium hydrate was used to dissolve 5 c.c. of five times concentrated precipitate.

TABLE IV.

Mouse.	Protective fluid.	Culture.	Result.
		Pneumococcus, type I, 100–4	
1	Original serum 0.2 c.c.	0.1 c.c.	Survived.
2	Original serum 0.2 c.c.	0.01 c.c.	Survived.
3	Original serum 0.2 c.c.	0.001 c.c.	Survived.
4	Precipitate emulsion, original volume 0.2 c.c.	0.1 c.c.	Died, 36 hrs.
5	Precipitate emulsion, original volume 0.2 c.c.	0.01 c.c.	Survived.
6	Precipitate emulsion, original volume 0.2 c.c.	0.001 c.c.	Survived.
7	Precipitate emulsion concentrated 5 times	0.1 c.c.	Survived.
8	Precipitate emulsion concentrated 5 times	0.01 c.c.	Survived.
9	Precipitate emulsion concentrated 5 times	0.001 c.c.	Survived.
10	Precipitate concentrated 5 times dissolved in sodium hydrate	0.1 c.c.	Died, 4 dys.
11	Precipitate concentrated 5 times dissolved in sodium hydrate	0.01 c.c.	Died, 40 hrs.
12	Precipitate concentrated 5 times dissolved in sodium hydrate	0.001 c.c.	Died, 13 hrs.
		Pneumococcus, type II, 34–7	
13	Precipitate emulsion concentrated 5 times	0.1 c.c.	Died, 15 hrs.
14	Precipitate emulsion concentrated 5 times	0.01 c.c.	Died, 15 hrs.
15	Precipitate emulsion concentrated 5 times	0.001 c.c.	Died, 18 hrs.
16	Precipitate emulsion concentrated 5 times	0.0001 c.c.	Died, 15 hrs.
		Pneumococcus, type I, 100–4	
17	Control	0.00001 c.c.	Died, 32 hrs.
18	Control	0.000001 c.c.	Died, 24 hrs.
		Pneumococcus, type II, 34–7	
19	Control	0.00001 c.c.	Died, 18 hrs.
20	Control	0.000001 c.c.	Died, 14 hrs.

This experiment indicates the protective value of undissolved precipitate emulsion, shows its specificity, and that concentration increases its potency. The deleterious effect of solution in sodium hydrate is also shown.

We are at present engaged in determining other methods of dissolving the precipitate which may have no effect on the protective bodies. Our experiments show further that the agglutinins as well as the protective bodies are brought down with the precipitate. Preliminary experiments indicate that a comparison of the protective value of precipitates with that of the original serum may be made more convincing by employing rabbits instead of mice. It is at least evident that a simultaneous injection of serum or of the precipitate will protect rabbits from an intravenous dose of the pneumococcus that is many times the fatal dose. The precipitates have shown themselves to be as protective as the original serum in these animals, although the end-point of the protective value has not been reached in our experiments to the present time.

TABLE V.

Protein Content of Antipneumococcus Serum before and after Precipitation, and of Its Precipitates with Indication of Protective Value of Precipitates.

Precipitate lot.	Protein content.		Exhausted serum.	Protective value of precipitate.
	Original serum.	Antigen.		
I^1				Protects well.
I^2 0.34%				Protects well.
I^3 0.18%	5.76%		7.0 %	Partial protection.[7]
I^4				Partial protection.[7]
I^5 0.09%	6.49%	0.015%	7.19%	Partial protection.[7]
I^6 0.108%				Protects well.
I^7 0.101%	5.9 %	0.022%	5.07%	Protects well.
II^3 0.21%	5.9 %		5.90%	Does not protect.
II^4 0.157%	5.56%	0.022%	5.72%	Protects well.

It remains to discuss the relative protein contents of serum and precipitate. Our results here are somewhat varying and fragmentary, but show that the protein content of the precipitate as compared with serum is surprisingly low. There would, moreover, seem to be no necessarily direct relation between protein content and protective value. We are indebted to Mr. Cullen for the Kjeldahl determina-

[7] The failure to demonstrate good protection in this serum is probably due to the fact that only the dissolved sodium hydrate precipitate was tested.

tions of total nitrogen content on which these figures are based. The estimates were made in duplicate and usually from five cubic centimeters each of a dilution of each fluid.

It will be noted from table V that in all instances of precipitates from antipneumococcus sera of type I, where only partial protection was obtained by the precipitates, the original precipitate emulsions were not tested, but only the precipitates after solution in sodium hydrate which, as has been evidenced, may destroy the protective power in precipitate or in serum.

The average protein content of more or less perfectly protective precipitates is, as will be noted, very low, ranging from 0.09 to 0.34 per cent., or from one-seventieth to one-thirty-second of the amount of protein in the original serum in those instances in which full computation was made. The obvious value of the possible employment of concentrated immune bodies in a solution of such low protein concentration for intravenous injection in human beings is apparent. The avoidance of serum sickness and of the possible formation of antagonistic bodies seems possible by this means. The advantage of this method of concentration of the protective bodies over concentration by chemical precipitation lies not only in the low protein content of the precipitate, but also in the fact that it may be produced rapidly and under conditions of absolute asepsis.

CONCLUSIONS.

The addition of a water-clear extract of pneumococci to homologous antiserum produces a voluminous precipitate which carries down with it the agglutinins and practically the totality of the protective bodies against pneumococcus infection in animals. This precipitate when washed and resuspended in saline solution to the original volume of serum protects as well as the whole serum. The protein content of such solutions has varied from 0.09 to 0.34 per cent., as contrasted with about 6 per cent. in the original serum. The solution of this precipitate is not necessary to insure protection, and when produced by dilute alkali (sodium hydrate) frequently destroys the immune bodies.

In conclusion the senior author wishes to express his appreciation of the courtesy which Dr. Cole has shown in placing the facilities of the hospital at his disposal for this work.

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